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(54) Title: METHODS FOR THE DIAGNOSIS OF CHRONIC LOWER BACK AND CERVICAL PAIN

(57) Abstract

Methods of diagnosing and monitoring chronic back and cervical pain are disclosed. The methods involved subjecting a body fluid sample from a patient suspected of having chronic lower back or cervical pain to two-dimensional electrophoresis or an immunoassay and measuring relative amounts of protein or proteins which increase or decrease in concentration as compared to a standard control. Also disclosed are kits for use with the diagnostic methods.

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TITLE OF THE INVENTION

Methods for the Diagnosis of Chronic Lower Back and Cervical Pain

FIELD OF THE INVENTION

This invention relates to novel methods for the diagnosis of chronic back and cervical spine pain.

BACKGROUND OF THE INVENTION

Eighty-five percent of the United States population, at one time or another, seek medical consultation for chronic back ache. Over 40 million people claim disability due to chronic back pain (or low back syndrome) and the medical costs alone to care for this group is over 40 billion dollars [Aronoff, G.M., Evaluation and Treatment of Chronic Pain, Urban and Schwarzenberg, Baltimore (1985)]. This does not include the enormous socio-economic loss, estimated to be in the trillions of dollars. In 1985, 2.7 million individuals received social security disability insurance at a overall cost of $ 18.9 billion [Social Security Administration, Report of the Commission on Evaluation of Pain, Washington D.C., Department of Health and Human Services (1986)].

Clinically, chronic pain, as opposed to acute pain, is continuous pain which persists for six months or more. Pain has been defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [International Association for the Study of Pain, chaired by Mersky (1979)]. Vasudevan has noted that there are several aspects to pain: nocioception (the perception of pain, a physical stimulus); interpretation of the stimuli as "painful"; and the evaluation of the pain as creating suffering. [Vasudevan et al., "Counseling the Patient with Chronic Pain--The Role of the Physician", In Persistent Pain, Kluwer Academic Publishers, Boston (1988)].

In the present state of the art, there is no objective, statistically significant test for cervical (neck) spine and low backache and/or radiating pain. More specifically, there is no protein-based clinical test that quantitatively detects the presence or absence of cervical spine and chronic back syndrome, much less one having the capability to quantitate or monitor the progression or regression of chronic lower back or cervical pain. The existence of such a test would be of infinite value to the patient, the doctor, and the society which bears the cost-burden of this problem. An objective test for chronic back and cervical pain would allow for the following:

1. Verify the presence or absence of low back or cervical syndrome based on an organic cause. Ideally, this test would be performed on the first visit so that a baseline could be established to take advantage of the quantitative aspects of the test. If no organic cause existed, according to the test, then it would not be necessary to proceed with the more costly examinations (e.g., MRI's, CT scans, myelograms, discograms, bone scans, electromyelograms,and consultations) that are, in the present state of the art, required to rule out causes for this syndrome. If an organic cause is determined to exist, then the routine work-up could proceed with high expectations for success. At this point, if the routine tests for chronic back syndrome are negative and the protein-based analysis disclosed herein is positive for back syndrome, then the treating physician would be justified in continuing to seek a correctable, organic cause.

2. Monitor the progress and effectiveness of treatment. All chronic back syndromes are treated conservatively, initially. The effect of this treatment could be assayed and one or more of the following judgments could be made: continue with an improving test; discontinue with a worsening test; change treatment with a worsening test; recommend initial or revision surgery only when the test was worsening or not showing any improvement. Thus, an objective, biochemical test would increase the efficiency of conservative patient management and eliminate any unnecessary

surgery. It is foreseeable that the test disclosed herein would become the standard for assessment, wherein surgery would be indicated only if the protein analysis indicated it.

3. Identify the point of maximal medical improvement. By periodically administering the test during a course of treatment, the quantitative characteristic of the test would allow the physician to assess the degree of the pain of backache and/or radiating pain and cervical spine pain and assist him in identifying the point where medical treatment should cease. At this point, treatment and rehabilitation efforts can stop and the physician, patient, and employer can feel comfortable with a recommendation to return to full-time work, limited work, settle claims, retirement, etc. Medical costs should be reduced while the efficacy of medical treatment improves.

4. Identify those patients who are suffering disability from the pain of neck pain, backache and/or radiating pain from those who are not suffering from the pain of neck pain, backache and/or radiating pain. (Radiating pain is defined as pain that is perceived in one or both buttocks and/or one or both lower extremities.) This will assist the proper authorities in placing those who qualify for financial assistance because of an objectively documented back pain condition in the appropriate social program, and to identify and remove those who do not medically qualify.

5. Aid the courts and others concerned with assessing correctly the compensable damages of pain and suffering secondary to cervical pain, backache and/or radiating pain.

Clinical tests for chronic back syndrome include inspection, palpitation and manipulation. The vast majority of clinical tests depends upon the patient reporting a painful or other type of response, and are therefore unreliably subjective. Objective clinical tests in the current state of the art include reflex changes, spasm and properly performed straight leg raising tests, and may or may not aid in the diagnosis of lower back syndrome. Moreover, they neither quantitate nor monitor the progression of

lower back syndrome.

Thermograms, psychological interviews (e.g., McGill and MMPI tests), polygraphs and instrumentation tests may also be used to assist in the diagnosis of chronic back pain. However, none of these is completely accurate because they are also subjective and depend on the patient reporting the type and degree of response sustained.

Laboratory tests such as X-rays, CT scans, MRI's, myelograms, discograms, EMG's and bone scans can only delineate the presence or absence of possible pain-producing lesions which must then be correlated with the clinical findings of chronic back syndrome. They do not detect the presence or absence of low back syndrome itself, or in any way quantitate it. Further, it is not uncommon to have false positive and false negative results with these tests. All or any of these tests may be negative and the patient may continue to complain; on the contrary, all or any of these tests may be positive and a patient may remain asymptomatic. Moreover, some of these tests expose the patient to unnecessary radiation.

The capacity to obtain diagnostic information from proteins, particularly blood proteins, has progressed rapidly since the middle of the 19th century when it was believed that serum contained but a single protein, albumin. By 1887, Lewith had demonstrated, by salt precipitation, that serum proteins could be separated into the albumins and globulins. The ratio of albumin to globulin (A/G ratio) was shown to have diagnostic value and is still in use today. With the introduction of electrophoretic separations, immuno-analytic techniques and enzymatic assays, the number of plasma proteins of diagnostic value has grown exponentially. The examination of specific blood proteins has proven to be an invaluable diagnostic aid , as in the monitoring of creatine phosphatase levels in determining cardiac damage following a myocardial infarct. The increased resolution and detection of plasma proteins with two-dimensional electrophoresis [O'Farrell, J. Biol. Chem., Vol. 250, pp. 4007-4021 (1975)] combined with silver-staining [Merril, Proc. Natl. Acad. Sci., USA, Vol. 76, pp. 4335-

4339(1979)] allows investigators an examination of over one thousand proteins in human plasma and approximately 300 proteins in human cerebrospinal fluid.

Anderson et al. [Proc. Natl. Acad. Sci., USA, Vol. 74, pp. 5421-5425 (1977)] initiated the mapping and the identification of the plasma proteins resolved by two-dimensional electrophoresis. The goal of this work was to use these proteins for screening genetic variants. By 1984, they were able to identify only 38 of 646 serum proteins visualized by their electrophoretic and staining systems [Anderson et al., Plasma Proteins, Vol. IV, pp. 221-269, Academic Press, New York (1984)].

It has been suggested that two-dimensional gel electrophoresis can be used to correlate the presence of a protein in serum or tissue, or an increase in its amount, with various diseases [Tracy et al., "Two-Dimensional Gel Electrophoresis: Methods and Potential Applications in the Clinical Laboratory", J. Clin. Lab. Autom., Vol. 3, No. 4, p. 235 (1983)]. It has also been noted that development of a protein "profile" for disease states may be useful in diagnosis [Tracy et al., supra at 242].

However, the increase in resolution provided by two-dimensional electrophoretic techniques and the increased detection available with recently developed staining methods has not yet resulted in widespread clinical applications of this methodology. Thus, the diagnoses of disease states in general, and chronic back pain in particular, by way of two-dimensional gel analysis is new, there being only one such reported method. This method utilizes two-dimensional gel protein analysis of cerebrospinal fluid to distinguish Creutzfeldt-Jakob disease from other causes of dementia [Harrington et al., U.S. Patent No. 4, 892, 814).

Harrington et al. [Clinical Chem., Vol. 31, pp. 722-726 (1985)] also found some proteins associated with Parkinson's disease and schizophrenia, which may or may not be of diagnostic value. Some proteins mapped and identified by two-dimensional electrophoresis of plasma [Anderson et al., 1984, supra] and cerebrospinal fluid [Goldman et al., Clin. Chem., Vol. 26, pp.

1317-1322 (1980)] have demonstrated to be polymorphic and thus may provide for genetic and forensic applications, but have not proven reliable as diagnostic markers for particular diseases.

To overcome the aforementioned deficiencies in the art, the present inventors have developed an objective, quantitative test for diagnosing chronic back syndrome and cervical spine pain. The test utilizes two-dimensional electrophoresis to analyze the increased or decreased concentrations of certain proteins in a body fluid sample from a patient as compared to a normal control.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide for an objective, diagnostic test for chronic cervical or lower back pain.

It is a further object of this invention to provide a method for determining the severity of chronic lower back or cervical pain.

It is yet a further object of this invention to provide for a method of determining the type (i.e., conservative versus surgery) and the effectiveness of a course of treatment for chronic lower back or cervical pain.

In the course of developing the foregoing objects, the present inventors have also identified proteins associated with chronic back pain and cervical pain according to their migration on two-dimensional polyacrylamide gels. Thus, the so-identified proteins and products derivable therefrom (for example, antibodies) are also part of the present invention. As one skilled in the art would recognize a spot on a stained two-dimensional gel could represent one or more proteins.

A number of proteins detectable by the methods of the present invention have been found to be altered in concentration and/or migration pattern. As would be expected in studies of this nature, some protein spots have been found to be of a higher diagnostic value than others when subjected to statistical analysis.

According to an initial study of the present invention, chronic lower back pain is accurately diagnosed by the detection of

increased or decreased levels of forty-four proteins in patients suspected of suffering from chronic back pain as compared with normal controls (i.e., normal volunteers with no clinical evidence of chronic back pain). In patients with chronic back pain, 29 proteins are found to be increased to levels at least three-fold as compared to normal controls, with statistical significance. (Statistical significance is defined herein as a p value of less than or equal to 0.05 by the Student t test or the log Student t test.) Of these 29 proteins, 13 are found to only occur in chronic back pain patients. On the other hand, patients with chronic back pain exhibit decreased levels of at least three-fold of 13 proteins as compared to controls. Seven of these proteins are totally absent in patients with the back pain syndrome. A further investigative study ("second study") found one additional spot which has very high predictability of the presence or absence of back pain or cervical pain and, thus, is favored for diagnostic value, as well as ease of observation.

Thus, the diagnostic methods of the present invention can employ techniques to identify the increase or decrease, or presence or absence, of these proteins in a sample to diagnose chronic back syndrome or cervical pain. Of course, the presence or absence of a defined protein or proteins in a spot in a specific location on the gel may be due to changes in the migration of proteins altered in charge or molecular weight. Also, one may perform multivariate analyses on an array of more than one of the proteins by usual statistical methods.

These proteins are identified by their relative molecular weight and isoelectric point (i.e., their migration on two-dimensional electrophoresis gels). The exact identity of the proteins is not yet known. However, as chronic lower back pain and cervical pain may be associated with inflammation, some of the proteins which appear to be increased in chronic back pain may belong to the class of plasma proteins which are known to be increased in response to tissue injury and disease. This class of proteins was first discovered by the observation of their induction

in patients with pneumococcus pneumonia [MacCarty, M., "Historical Perspective on C-Reactive Protein.", In Kushner et al. (Eds.,) C-Reactive Protein and the Plasma Protein Response to Injury, Annals of the New York Academy of Sciences, Vol. 389, pp. 1-10 (1982)]. Since these initial observations, the metabolic and physiological changes that occur in the acute phase response have been studied in numerous laboratories. It has been found that the acute phase response may be invoked by many different types of stimuli, such as trauma, infections, noninfectious inflammatory states, and tissue infarctions. See, Kushner et al. (Eds.), supra. While it is known that most of these proteins are synthesized in the liver, the nature of their induction is not yet known. Induction could be by blood borne substances or by neuronal factors since there are both blood vessels and nerves in the region of synthesis, the hepatic lobes [MacIntyre et al., "Biosynthesis of C-Reactive Protein.", In Kushner et al. (Eds.), supra, pp. 76-87].

Some of the acute phase response proteins have been induced in mice, and their relative positions have been identified with two-dimensional electrophoresis [Pluschke et al., Clin. Exp. Immunology, Vol. 66, pp. 331-339 (1986)]. The present applicants could not be sure that the proteins affected in the present invention are the previously observed acute phase response proteins or, perhaps, new members of this class of response proteins. However, the applicants sent an aliquot from each of the clinical samples to a commercial laboratory for measurement of complement C3, alpha-1 antitrypsin, transferrin, alpha-1 acidic glycoprotein, and C-reactive protein (some of the well characterized acute phase response proteins). No elevation of these proteins could be detected by standard assays. However, this may be more a reflection of the sensitivity of the assay rather than a lack of variation in the acute phase response proteins. On the other hand, it also may be postulated that certain of these proteins are apolipoproteins which are well documented to accumulate markedly during the regeneration of damaged peripheral nerves (less in the regeneration of damage to the CNS). In fact, preliminary studies

with Western blots have shown that lbp13.14.719 gives a positive result with anti-ApoE serum; however, this may be due to cross-reactivity.

It is unlikely that the protein changes noted herein are due to drugs, such as those the patients may have taken to alleviate their pain, since three of the patients in the initial study were not taking any medication for their chronic back pain. These three patients displayed protein alterations that were similar to those taking medication. It is also unlikely that the protein changes are artifacts of storage. Tracy et al. demonstrated the occurrence of plasma proteins which are altered by freezing and storage at -20°C [Tracy et al., Clin. Chem., Vol. 28, pp. 890-899 (1982)]. Our spots 1305, 1318, 1323 and 4614 are in the region noted for the appearance of such spots by Tracy et al. However, as the patient and the age and sex matched control samples were drawn at the same time and stored under identical conditions, it is unlikely that the proteins of interest in this study are storage artifacts.

DESCRIPTION OF THE DRAWINGS

Figure 1: Plasma gel stained with silver. This gel was made with 1.47 ul of plasma from a control. Circles mark proteins not generally visible in controls but present in patients with chronic back pain. Numbers preceded by 'M' designate landmarking proteins identified in Table I. The remaining labeled proteins are those which either increased or decreased by a factor of three or more and were statistically significant, as indicated in Tables II and III.

Figure 2: A computer generated diagram of all the proteins analyzed in [this] the initial study. The numbered proteins are the same as those identified in Figure 1.

Figure 3: A scatter diagram illustrating proteins ,which showed robust correlations with chronic back pain in the initial study. The numbers to the left of the circle indicate the frequency greater than 1 that a patient with that value was observed. The circle and bar to the right of each group of data

indicate the mean and the standard error of the mean for that group.

Figure 4: A scatter diagram illustrating proteins which showed moderately robust correlations with chronic back pain in the initial study. The numbers to the left of a circle indicate the frequency greater than 1 that a patient with that value was observed. The circle and bar to the right of each group of data indicate the mean and the standard error of the mean for that group.

Figure 5: A patient by patient comparison of protein 1318 densities with the degree of lower back disability. The degree of disability was scored by using a number of factors, such as: measurements of back and leg motion limitations, abnormalities in the knee jerk reflexes and history of back surgery.

Figure 6: A patient by patient comparison of protein 1316 densities with the degree of lower back disability.

Figure 7: A patient by patient comparison of protein 1204 densities with the degree of lower back disability.

Figure 8: A patient by patient comparison of protein 1305 densities with the degree of lower back disability.

Figure 9: A patient by patient comparison of protein 3203 densities with the degree of lower back disability.

Figure 10: A patient by patient comparison of protein 3211 densities with the degree of lower back disability.

Figure 11: A patient by patient comparison of the average of proteins 1316 and 1318 densities with the degree of lower back disability.

Figures 12-14: 2-D gel images of three patients with chronic lower pain back in the second study, showing the spot lbp13-14.719.

Figures 15-17: 2-D gel images of three controls run side-by-side with the gels of Figures 12-14. Open circles represent area where lbp13-14.719 spot is missing.

Figures 18-20: Represent enlargements of the areas blocked off in Figures 12-14.

Figures 21-23: Represent enlargements of the areas blocked

off in Figures 15-17.

DETAILED DESCRIPTION OF THE INVENTION

This invention involves methods of diagnosing chronic lower back or cervical spine pain wherein protein samples from both normal and abnormal individuals are subject to two-dimensional electrophoresis and/or immunoassays. In the case of two-dimensional gel electrophoresis, a large number of protein spots common to both types of individuals and spots which appear or disappear in the abnormal patient group are determined. Initially, the number of protein spots to be examined is reduced to only those showing statistically significant differences between normal controls and patients with chronic back pain. This is determined by performing a Student's t test or a log Student's t test on the spot intensity data. Those proteins that have statistical differences at a significance level of 0.05 on either or both of these tests are chosen for further study.

Any number of protocols can be used to develop protein data for use in performing the diagnostic methods of the present invention. The protocol used in the present studies and as exemplified herein was approved by the IRB of St. Luke's Hospital in Houston, Texas, and patients and sex and age matched volunteers each of whom signed an informed consent letter. The patients were complaining of chronic (six months or more in duration) low back pain secondary to a reported injury and were randomly selected from one of the applicants' orthopedic practice. The patients were requested to remain drug-free for at least one week prior to blood sampling. The controls were free of significant medical problems as determined by medical history and physical examination. The initial study consisted of 10 patients and 10 sex and age matched controls. In the second study, a first analysis consisted of 64 plasma samples from 32 lower back pain patients and 32 controls, and a second blind analysis consisted of 17 plasma samples from 10 lower back pain patients and 7 controls.

To prevent degradation of samples of tissue, serum, or other

body fluids (preferably blood and more preferably plasma) from the subjects, the sample is initially frozen in dry ice. At any point prior to electrophoresis a portion of the sample may be removed for counting and assaying the amount of protein by, for example, the Lowry method.

The first stage gels for the two-dimensional electrophoresis generally contain urea at a concentration of about 9M and about 2% nonionic detergent, both of which aid in dissociating proteins. The nonionic detergent helps keep the separated proteins from precipitating at their isoelectric points. These reagents and their proportions can vary somewhat, provided that these objectives are accomplished. An ampholyte (e.g., 2% 4-8 pH ampholyte) is also desirable to maintain a pH gradient across the length of the gel, although other reagents which maintain the pH gradient could be substituted. The acrylamide concentration of the first stage gel should be such as to permit protein movement to the isoelectric point. The first stage gel can be in a number of forms; for example, it can be housed in an isoelectric focusing tube or in a slab form. Preferably, the first dimension is in the form of a tube gel.

The samples are usually prepared for the first stage by solubilizing in either sodium dodecyl sulfate (SDS) or urea at a concentration of about 9M. The reducing agent 2-mercaptoethanol is also usually included to separate disulfide-linked subunits. An ampholyte to maintain the pH gradient, a nonionic detergent which does not affect the protein charge, and dithiothrietol (DTT) which disrupts disulfide bonds, may also be included. Other reagents may also be added, or other reagents which accomplish the foregoing functions may be substituted. For example, prior to subjecting the samples to gel electrophoresis, the samples may be placed in a sample buffer (preferably ~0.3% SDS, 5% 2-mercaptoethanol and TRIS buffer, pH~8) and placed in a boiling water bath (at about 100°C) for about two minutes to aid in dissolution. This temperature and the time exposed thereto has been found to not cause protein degradation; however, both can be varied provided that dissolution

takes place and protein degradation does not. The sample buffer unfolds the protein, separates the disulfide-linked subunits, and maintains the pH. Other sample buffers which accomplish the foregoing functions, and other methods of dissolving the protein samples, can also be used.

The samples may then be cooled on ice and treated with DNase and RNase to reduce the viscosity. The samples can then be snap-frozen in liquid nitrogen and packed on dry ice if they are not to be run on gels immediately. This adequately preserves the samples. However, it has been found that any method that cools a dissolved sample to -70^{o}C or more will also preserve the samples.

Polyacrylamide gel electrophoresis in the presence of SDS is usually used for the second dimension separation. SDS is an ionic detergent and binds strongly to proteins. It eliminates the native protein charge characteristics and unfolds the protein into a rod-like form. See Tracy et al., J. Clin. Lab. Autom., supra. Thus, when protein is subjected to an electric field in the polyacrylamide gel matrix, the lack of charge and the relatively uniform shape of the SDS-protein complexes allow separation essentially by molecular weight, with the polyacrylamide gel matrix acting as a sieve.

The second stage gel is preferably SDS-equilibrated, to eliminate the protein charge, and contains a higher acrylamide concentration than the first stage gel, to aid in separating proteins by molecular weight. Other reagents can be added or substituted. Further, the second gel can be in a variety of forms; preferably, however, the second stage is in the form of a slab gel.

The spots on the gels can be viewed by any number of methods including staining with Coomassie blue and silver staining. They can be visualized for relative protein density manually, but it is preferred that they be scanned with an appropriate camera system with a normalization standard (available from the National Bureau of Standards, Gaithersburg, Md.) and analyzed with a computer densitometer to measure relative protein spot staining intensities or densities.

In order to perform immunological tests for the diagnosis and/or monitoring of chronic lower back syndrome or chronic cervical pain, the first step is to obtain antibodies to the proteins of interest. There are many methods of accomplishing this which are well known to those skilled in the art. (For comprehensive laboratory methods, see Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988), which is incorporated by reference herein.) For antibodies with sufficient specificity for western blots and immunoassays, the antigen must be purified to homogeneity or the antigen should be used to prepare monoclonal antibodies. Since the proteins of interest in this invention are seen as unique spots on the second dimension polyacrylamide gel, preferably the gel can be used as the final purification step of the individual antigens. One can obtain a pure antigen preparation by excising the spots which show increased or decreased intensity in chronic lower back pain patients. This gel piece can be injected into an animal to raise antibodies. Alternatively, one may cut out the spot of protein and electroelute it from the gel to obtain a protein in solution for injection. Still another technique for processing the protein for injection after separation on gels is to electrophoretically transfer the proteins to nitrocellulose, locate the proteins of interest by staining (e.g., with Ponceau S), excise the spots and cut into pieces for injection. The particular method used is only limited by the ability to elicit an immune response to the proteins of interest.

As an alternative to using the antigen purified by separation on an electrophoretic gel directly, one may elute a protein spot, obtain a partial sequence by any method well known in the art, and use the sequence to manufacture synthetic peptides (usually with an automated machine using solid-phase techniques). The synthetic peptide should be at least six amino acids long to elicit antibodies that bind to the original protein. The purified synthetic peptides would then be coupled to carrier proteins, and these conjugates are then used to immunize animals.

The polyclonal antibodies in the antisera obtained with the foregoing methods can be used for western blots and other immunological tests. However, one may further utilize hybridoma technology to obtain monoclonal antibodies, which may be the best choice for immunochemical techniques. Methods of monoclonal production are well known in the art and were first described by Kohler and Milstein in 1975. Briefly, antibody-secreting cells are fused to, for example, myeloma cells to create hybridoma cells which are cloned and screened by appropriate methods for the desired antibodies.

One of the diagnostic methods of the present invention involves the detection of proteins which are present in patients with chronic back or cervical pain, yet absent in normal controls. Besides locating these protein spots by staining on a two-dimensional gel, one may detect the proteins by immunoblotting, or western blotting, utilizing the polyclonal or monoclonal antibodies raised to the particular protein of interest. Protocols for immunoblotting are well known in the art and generally comprise the steps of gel electrophoresis, transfer, blocking, addition of antibody, and detection. The preparation of the sample and the two-dimensional gel electrophoresis is discussed above. At the completion of electrophoresis, proteins are transferred from the gel to a matrix, such as nitrocellulose, activated (diazo groups) paper and activated (positively charged) nylon. Nitrocellulose membranes are preferred for relatively low background and cost considerations; however, any membrane which will sufficiently bind the transferred proteins can be used. Preferably, transfer of the proteins is accomplished by electrophoretic elution; however, simple diffusion or vacuum-assisted solvent flow can also be used. After transfer, the membranes may, optionally, be stained to determine the position of molecular weight markers.

Prior to antigen detection, one must block the membrane to prevent non-specific adsorption of immunological reagents. Most preferably, the blocking solution would be composed of nonfat dried milk or bovine serum albumin. After blocking, antigens can be

detected directly or indirectly. Direct detection utilizes labelled primary antibodies. The antibodies, labelled with iodine, enzymes or biotin, can be prepared by methods well known to those skilled in the art. In indirect detection, the primary antibody (unlabelled) is first added to membrane, followed by a secondary antibody (an anti-primary antibody) which is labelled with radioactive iodine or an enzyme, such as horseradish peroxidase. The antigen is then detected by exposing a radiolabeled membrane to X-ray film or, in the case of enzyme-labelled antibody, by adding substrate to the membrane.

An alternative method of quantifying or detecting the presence of protein for the diagnosis of chronic lower back or cervical pain is the use of immunoassays performed directly on the body fluid sample. Several immunoassays would be useful in the context of the present invention, including: antibody capture (Ab excess); antigen capture (antigen competition); and the two-antibody sandwich technique. All immunoassays rely on labeled antigens, antibodies, or secondary reagents for detection and quantitation. The label used can be radioactive or enzymatic, or one may label with fluorochromes or biotin. The choice of label is a matter of discretion with the diagnostician, taking into consideration cost, sensitivity, radioactivity exposure, etc. The term "label" as used herein refers to any of the foregoing.

In an antibody capture type of assay, the test sample is allowed to bind directly to a solid phase and any unbound antigen is washed away. The antibody specific for the antigen is added and allowed to bind. The amount of antibody bound to the solid phase, after washing away unbound antibody, is determined using a secondary reagent. Suitable secondary reagents include anti-immunoglobulin antibody, protein A or protein G. These can be obtained from commercial sources or prepared by methods known in the art. Detailed protocols can be found in Harlow et al., supra, and are incorporated herein by reference, the particular methods used not being limited or essential to the practice of the present invention.

Antigen capture type assays measure the amount of antigen in a test sample via a competition between labeled and unlabeled antigen. This type of assay is exemplified by a "radioimmunoassay" or RIA. The first step in this type of assay is to bind unlabeled antibody to a solid support (either directly or through an intermediate protein, e.g., an anti-immunoglobulin antibody). A sample of known antigen of known quantity is labeled and a sample of this is added to the test material containing an unknown amount of antigen, and the mixture is added to the bound antibody. The antigen in the test sample competes with the labeled antigen for binding to the antibody bound to the solid support. Following removal of the unbound antigen, the amount of labeled bound antigen is measured. The higher the concentration of antigen in the unknown test sample is, the more effectively it competes with the labeled antigen; therefore, a decreasing amount of label is detected with an increasing amount of unlabeled antigen. Thus, generated standard titration curves will yield relative levels of antigen.

Another immunoassay to quantitate antigen concentration is the two-antibody sandwich technique. This type of assay requires two antibodies that bind to two separate epitopes of the antigen. Thus, one may use two monoclonals that recognize two separate sites on the antigen, or a batch of purified polyclonals can be used. The essential steps are as follows: 1) one purified antibody is bound to a solid phase and the antigen in the test sample is allowed to bind to the first antibody; 2) unbound antigen is washed away and a labeled second antibody is allowed to bind to the antigen; and 3) after washing, the second labeled antibody that is bound to the matrix is quantitated. As in other assays, a standard titration curve with known dilutions is plotted and the unknown sample is compared thereto. In order to determine absolute amounts, a standard curve generated with known quantities of antigen is used.

A wide variety of test kits are possible to take advantage of the advances in the diagnostic arts made possible by this

invention. Some will be described here; others can be devised by those skilled in the art.

The central reaction in a test kit could be between any one of the aberrant proteins found in patients with chronic lower back or cervical pain and the antibodies prepared as set forth above and in the Examples below. The Examples below are directed to the preparation of antibody from rabbits, and the following description and other Examples of test kits and test methods will be based on the rabbit preparation. The rabbit is the preferred source of immunoglobulin and its fractions; however, the skilled artisan will recognize that the following Examples utilize the rabbit only as exemplary. Other animals can be used and this will require some modification of the other reagents used in the tests and the kits, and are readily apparent to one skilled in the art.

In the test kits, any of a variety of adsorbents can be used including, for example, glass or plastic surfaces which may be the inner surfaces of test tubes or the surfaces of test plates. Examples of flat surfaces especially useful in an enzyme-linked immunosorbant assay (ELISA) or a radioimmunoassay (RIA) include glass, nitrocellulose paper, or plastics such as polystyrene, polycarbonate or various polyvinyls. The ligands can be attached to the surface by direct adsorption, forced adsorption and coupling, in accordance with known procedures. Typical test kits are set forth in the Examples below.

The following Examples illustrate the utility of the diagnostic methods of the present invention, and are not intended to limit the scope of this invention. For instance, any of the known immunoassays may be used to aid in the diagnosis of lower back or cervical pain. Also, the method of diagnosis is not limited to the specific proteins elucidated by the present Examples. Modifications of the procedures as would be apparent to one skilled in the art are within the scope of the teachings.

EXAMPLE 1

In a first (initial) study, ten patients with chronic back pain were randomly selected from a group of patients complaining of low back pain of six months or more secondary to a reported injury. These patients, 3 females and 7 males, ranged in age between 20 and 55 years. Of these patients, 7 were taking medication for their pain. However, 3 of these patients took no medication. The degree of lower back disability was evaluated by a number of factors such as: the history of the back pain (including radiations and the induction of pain with coughing and/or sneezing); physical examination including the loss of sensitivity in the L-4 to L-5 dermatomes; measurements of back and leg motion limitations; abnormalities in the knee jerk reflexes; and the analysis of spinal radiographies (for spondylosis, stenosis, herniated discs, degenerations, the narrowing of the intervertebral space, etc.)and other special studies such as MRI, CT scan, myelograms, discograms and electromyelograms. Controls were selected for age and sex to match the patient group. The controls were free of significant medical problems as determined by medical history and physical examination.

Ten ml of blood was collected by venipuncture, within one minute of tourniquet application, using vacutainer tubes containing 143 USP units of heparin. The control samples were collected at the same time as those from the patients and all samples were collected during afternoon hours. Plasma was isolated by centrifugation of the whole blood at 2000 x g for ten minutes followed by the separation of the plasma from the packed red and white cells by pipetting. The plasma was frozen at -20°C prior to shipment (in dry ice) to the laboratory for analysis. The samples were stored at -70°C until electrophoresis, which was performed within three months of venipuncture.

Gel electrophoresis. Plasma samples were thawed and 20 ul of each sample were added to 20 ul of denaturing solution, containing 10% w/v SDS and 2.3% DTT w/v. The samples were then heated to 95°C for 4 minutes followed by cooling to room temperature. Then 96 ul

of electrophoresis solution, containing 0.1 g DTT, 0.4 g CHAPS, 5.4 g urea, 0.5 ml pH 3.5-10 ampholytes and 6.5 ml deionized water were added to each sample. The samples were mixed on a Vortex mixer and 10 ul of each processed sample (containing 1.47 ul plasma) were added to the first dimension isoelectric focusing (IEF) gels. Isoelectric focusing was performed in 3% (w/v) acrylamide gels with 4% w/v ampholytes (containing a 1:1 mixture of pH 3.5-10 and pH 5-7 ampholytes) and crosslinked with 0.03% diacrylpiperazine (PIP or DIP), i.e., 3%T/1%C. Electrophoresis was performed for 18,000 volt hours, beginning with 1000 volts for 17 hours followed by 2000 volts for 30 minutes.

The second dimension, wherein proteins are separated by mass, was performed with 160 cm x 200 cm x 1.5 mm slab gels using a Bio-Rad Protean II chamber. These gels were formed with 12.2% acrylamide (w/v), 0.2M TRIS-HCl (pH 8.8), 0.7% sodium thiosulfate (w/v), 0.3% diacrylylpiperazine (w/v), 0.5% 1,4-dimethylpiperazine(v/v), and 0.07% ammonium persulfate (w/v). Electrophoresis was performed at 7°C with a constant current of 40 mA per gel.

Silver staining. At the end of the run, the gels were removed from the glass plates and washed for 5 minutes in water (no protein loss is detected during this period). The gels were then soaked in a solution of ethanol/acetic acid/deionized water (40/10/50) for one hour on an orbital shaker at 36 rpm. This solution was then replaced with a solution of ethanol/acetic acid/ deionized water (5/5/90) and the gels were soaked for at least 3 hours. The gels were washed with deionized water for 5 minutes and soaked in gluteraldehyde solution (100 ml/liter) for 30 mintues. Extensive washes with deionized water were performed to entirely remove the gluteraldehye3 X 10 and 4 X 30 minutes). [Cold deionized water (<15°C) removes gluteraldehyde more efficiently.] The gels were then stained for 10 minutes in an ammoniacal silver nitrate solution (6 g of silver nitrate dissolved in 30 ml of deionized water, which is slowly mixed into a solution containing 160 ml of water, 10 ml of concentrated ammonium hydroxide, and 1.5 ml of

sodium hydroxide, 10 mol/liter; this solution is then diluted with deionized water to a final volume of 750 ml) (solution H)> The temperature of solution H was 20°C. After staning, the gels were washed with deionized water for 5 minutes X 3. The image was then developed in a citric acid and formaldehyde solution (0.1 g citric acid and 1 ml formaldehyde in 1 liter of deionized water) (solution V) until a slight background stain appeared. (The optimum temperature of solution V is 15-18°C.) The development process was stopped with an acetic acid /deionized water solution (5/95) for at least 15 minutes. Stained gels were stored in a glycerol/ethanol/ deionized water solution (7/10/83). [This staining method is described in Hochstrasser et al., Analytical Biochemistry, 173, pp. 424-435 (1988).]

Gel analysis. In order to quantitate proteins, gels were scanned with an Eikonics Series 78/99 digital scanner and the gel images created thus were analyzed using PDQUEST software (Protein Database, Inc., Huntington Station, NY) on a SUN 4-260 minicomputer. Gel images were normalized for protein loading and staining variation using the average log-ratio normalization procedure of the PDQUEST software. Thirteen hundred proteins were analyzed on each gel and the proteins were matched and compared quantitatively. This analysis was performed by using the PDQUEST software aided by visual examination and operator intervention in gel areas containing complex spot patterns. All proteins which increased or decreased in concentration by three-fold or more, and were found to be statistically significant (by way of the Student t test or the log Student t test) were considered to be spots of interest.

Figure 1 represents a stained gel of a plasma sample from a control subject. Marker proteins are designated by the prefix M, and Table I lists the marker plasma proteins landmarked on the gel. Proteins which were found to be increased or decreased in patients with chronic back pain are listed in Tables II and III, respectively, and are indicated by number on the gel shown in Figure 1. Those proteins which are absent in patients' plasma are

illustrated by open circles on the control gel in Figure 1. A computer-generated master map of the proteins analyzed in this Example is represented in Figure 2.

Table I

Landmarked Plasma Proteins

Identification No.	Protein
M1	beta-Haptoglobins
M2	$alpha_1$-Antitrypsins
M3	Albumin
M4	IgG heavy chains
M5	Apo A-1 lipoproteins

Table II

Plasma Proteins Increased With Chronic Lower Back Pain

Protein ID #	Controls (N = 9) Mean Conc.*	Controls S.E.M.	Controls Frequency	Patients (N = 10) Mean Conc.*	Patients S.E.M.	Patients Frequency	fold Increase or decrease
4210	0.0	0.0	9	51.5	24.2	5	>51.5
1204	0.0	0.0	9	46.5	12.3	8	>46.5
1318	0.0	0.0	9	42.5	15.3	7	>42.5
1324	0.0	0.0	9	38.3	14.7	6	>38.3
1702	0.0	0.0	9	31.2	13.9	4	>31.2
4821	0.0	0.0	9	31.2	16.6	4	>31.2
0508	0.0	0.0	9	26.4	9.2	5	>26.4
3211	0.0	0.0	9	25.5	9.7	5	>25.5
4109	0.0	0.0	9	21.9	10.1	4	>21.9
5524	0.0	0.0	9	20.2	10.5	4	>20.2
7622	0.0	0.0	9	19.8	8.2	4	>19.8
6331	0.0	0.0	9	13.0	5.4	4	>13.0
8322	0.0	0.0	9	10.1	4.5	4	>10.1
3151	1.6	1.6	1	46.8	15.2	7	29.3
1323	2.0	2.0	1	50.0	18.5	6	25.0
5240	3.4	3.4	1	69.2	30.7	6	20.3
2105	2.0	2.0	1	31.5	11.8	6	15.8
1316	4.9	4.9	1	75.2	18.1	9	15.3
6104	7.4	4.7	2	94.5	41.2	6	12.8
7109	18.5	12.3	3	131.2	42.9	6	7.1
3606	63.7	30.5	4	402.8	130.0	8	6.3
1305	17.3	7.4	5	103.8	28.0	9	6.0
3146	3.7	3.7	1	21.0	4.8	7	5.7
7114	23.3	15.5	2	118.2	38.5	7	5.1
7448	67.7	46.3	3	283.4	79.4	9	4.2
3203	4.2	4.2	1	17.5	8.2	6	4.2
0609	37.3	19.1	3	148.3	46.5	8	4.0
0604	62.9	29.0	4	236.0	61.5	9	3.8
7711	15.4	15.4	1	49.4	17.3	6	3.2
5615	89.1	89.1	1	226.8	64.9	8	2.5

* Concentrations are in arbitrary density units.

p is less than or equal to 0.05

Table III

Plasma Proteins Decreased In Chronic Lower Back Pain

	Controls (N = 9)			Patients (N = 10)			Fold increase or
Protein ID #	Mean Conc.*	S.E.M.	Frequency	Mean Conc.*	S.E.M.	Frequency	decrease
4737	224.0	152.1	5	0.0	0.0	10	>224.0
4624	112.5	52.0	4	0.0	0.0	10	>112.5
7212	78.1	28.1	6	0.0	0.0	10	> 78.1
7309	13.0	5.8	4	0.0	0.0	10	> 13.0
7214	11.7	6.0	3	0.0	0.0	10	> 11.7
2806	11.6	4.7	4	0.0	0.0	10	> 11.6
5013	10.8	5.6	3	0.0	0.0	10	> 10.8
4614	12.5	4.4	6	2.3	1.6	2	5.4
3155	76.0	28.5	7	16.5	9.1	4	4.6
8309	53.8	11.0	9	13.1	4.7	5	4.1
8418	744.1	185.6	8	191.1	108.6	5	3.9
7455	95.6	26.8	7	25.5	10.9	4	3.7
5411	79.4	23.4	7	25.7	13.2	3	3.1
7515	8.2	2.6	6	3.2	1.9	3	2.6

* Concentrations are in arbitrary density units.

p is less than or equal to 0.05

EXAMPLE 2

In a further, expanded study ("second study"), 32 patients with chronic low back pain and 32 controls, were subjected to the methods set forth in Example 1. In an effort to identify new spots, the patient gels were compared to control gels visually without the aid of computer analysis. It was observed that one particular protein(s) spot appeared in patients and was virtually absent in controls. It is postulated that the computer did not identify this spot because it was incorporating it into another, very closely located spot on the gel. This new spot has been designated lbp13-14.719. Its presence is indicated on Figures 12-14 and 18-20, and the corresponding absence of the spot on the control gels is indicated by an open circle in Figures 15-17 and 21-23.

A summary of the data obtained in this study is shown in Table IV. The numbers under the "run" column are randomly assigned numbers which refer to four separate batches of gels run on different days. This study was "blind" to the extent that the investigator performing the analyses was unaware which gels were of back pain samples and which were controls. As can be seen from Table IV, the accuracy in predicting which samples belonged to which group is remarkably high (97% overall). Moreover, this new spot allows for visual identification without the need for computer-assisted analysis, thereby simplifying the diagnostic procedure.

Table IV
Back Pain Study of Example 2

Run No.	Patients Positve ID of Spot per no. of gels	Controls Absence of Spot per no. of gels	% correctly identified overall
lbp13	9/9	9/9	100
lbp14	7/8	7/7	93
lbp20	9/10	7/7	94
lbp21	5/5	9/9	100
Totals:	30/32	32/32	97

p is less than 0.005 by chi-squared analysis.

Following the above analysis, a blind study was conducted with 17 plasma samples from 10 patients and 7 controls in the same manner. The results of this study showed that nine out of ten (9/10) (90.00%) lower back pain patients, and all of the asymptomatic controls (7/7) (100%) were correctly identified on the basis of the presence or absence of lbp13-14.719. The combined accuracy was 16/17, or 94.12%, where p is less than 0.005 by chi-squared analysis.

It is possible that the spot, lbp13-14.719, represents more than one protein; however, based on the separation methods used here, it appears as a single spot.

EXAMPLE 3

Five additional groups of patients' plasma were analyzed with respect to lbp13-14.719. These groups consisted of:

A. Fifteen patients (nine males and six females) who had pain elsewhere than the lower back.

B. Five patients (all males) who had had previous backache, based on abjective findings, or who had documented biomechanical deficits but had no pain.

C. Seven patients (one male and six females) who had a chronic inflammatory disease.

D. Four patients (three males and one female) with periferal nerve lesions.

E. Three patients (all females) who had chronic cervical spine pain.

The above samples were subjected to the methods and analyses of Example 2, wherein the presence or absence of lbp13-14.719 was determined.

The results of group A are shown in Table V. Ten samples showed no lbp13-14.719. Five were positive, which indicated that there was nerve damage with repair. Two of these five had spine problems, two had severe fractures of the tibia and fibula with probable nerve damage, and one shoulder pain with an "adventitious bursa". The exact pathology of the last patient is not known, and

may include nerve damage. This demonstrated that the lbp13-14.719 is not exclusively confined to spinal pathology, adn that nerve damage elsewhere could produce a false positive. It also showed that nerve damage is present more than is suspected in some of these conditions.

The results of group B are shown in Table VI. The results show that two painless spondylolistheses and three operated backs that were asymptomatic showed the absence of lbp13-14.719 because there was no nerve damage or repaiar. This proved that presence of lbp13-14.719 was not a post-operative finding following the scarring of surgery, nor was it routinely present with the trauma of altered biomechanics. Nerve damage and repair were probably necessary to produce lbp13-14.719 in the plasma.

The results of group C are shown in Table VII. This study was performed to rule out the supposition that lbp13-14.719 was an acute phase protein. Lbp13-14.719 was present in three cases of Crohn's disease and two cases of lupus, but absent in two cases of rheumatoid arthritis. It may be that there is nerve damage in Crohn's disease and lupus, because the inflammation of rheumatoid arthritis does not produce the protein spot. These diseases may produce false positives.

The results of group D are shown in Table VIII. Three patients were positive for lbp13-14.719 and one patient having painless, radial nerve palsy was negative. The radial nerve in the arm is documented to recover poorly, and this absence of lbp13-14.719 may be an indication of an inadequate physiological repair. These conditions may give false positives when studying chronic lower back pain.

The results of group E are shown in Table IX. All three patients with chronic cervical spine pain showed positive for lbp13-14.719. This indicates that chronic cervical spine pain has the same biochemistry as chronic lower back pain, and that the lbp13-14.719 may be helpful in managing this condition. However,

TABLE V-GROUP A

PATIENT	AGE	SEX	DIAGNOSIS	lbp13-14.719
MB	68	F	S/P TOTAL HIP REPLACEMENT: LUMBAR SPONDYLOSIS	-
MO	59	M	FAILED TOTAL HIP REPLACEMENT	-
OB	22	M	EARLY ASEPTIC NECROSIS	-
SA	41	M	DETACHED DELTOID; CERVICAL SYNDROME	+
LH	34	M	S/P LIGAMENT TEAR, SHOULDER	-
JP	58	M	FAILED TOTAL HIP REPLACEMENT; MULT. COMP. FX'S	+
SH	59	F	SHOULDER PAIN: SCOLIOSIS W/LUM. SPONDYLOSIS	+
RG	15	M	RIGHT KNEE SPRAIN	-
SH	46	F	ADVENTITIOUS BURSA, LEFT SHOULDER	+
DC	34	M	ASEP. NEC.,LF HIP;DISLOC. STERNOCLAVICULAR JT.	-
DJ	47	F	SHOULDER PAIN	-
BC	34	F	FRACTURED RIGHT ULNA	-
WJ	40	M	ASEPTIC NECROSIS, BILATERAL HIPS	-
DS	47	M	ARTHRITIS, LEFT KNEE; FAILED INTERNAL FIXATOR	+
MW	29	F	SYN. & MEDIAL MENISCUS TEAR;S/P ARTHROSCOPY	-

"+" indicates presence of lbp13-14.719

TABLE VI-GROUP B

PATIENT	AGE	SEX	DIAGNOSIS	lbp13-14.719
BMC,SR	67	M	SPONDYLOLISTHESIS, L4-5 W/OBLITERATE OF L5-S1	-
PP	49	M	HNP L5-S1	-
CV	59	M	S/P LUMBAR LAMINECTOMY & FUSION	-
GV	44	M	S/P LUMBAR SURGERY	-
BMC,JR	45	M	SPONDYLOLISTHESIS, L5-S1	-

TABLE VII-GROUP C

PATIENT	AGE	SEX	DIAGNOSIS	lbp13-14.719
LJ	32	F	CROHN'S DISEASE	+
KC	27	F	CROHN'S DISEASE	+
LG	36	F	CROHN'S DISEASE	+
EH	33	F	LUPUS	+
GG	33	F	LUPUS	+
VC	60	F	RHEUMATOID ARTHRITIS	-
CE	76	M	RHEUMATOID ARTHRITIS	-

"+" indicates presence of lbp13-14.719

LEGEND OF ABBREVIATIONS

AR	ARACHNOIDITIS
CFX	COMPRESSION FRACTURE
DDD	DEGENERATIVE DISC DISEASE
FA	FACET ARTHROPATHY
FD	FACET DAMAGE
FF	FAILED FUSION
HNP	HERNIATED NUCLEUS PULPOSUS
IMP	IMPINGEMENT
INS	INSTABILITY
LST	LUMBAR STENOSOS
MBP	MECHANICAL BACK PAIN
PS	PSEUDOARTHROSIS
SB	SPINA BIFIDA
SC	SCOLIOSIS
SP	SPONDYLOSIS
SPT	SPONDYLOLISTHESIS
TV	TRANSITIONAL VERTEBRA

TABLE VIII-GROUP D

PATIENT	AGE	SEX	DIAGNOSIS	1bp13-14.719
MW	24	M	MULTIPLE FRACTURES; NERVE PALSY	–
EP	42	F	CUBITAL TUNNEL SYNDROME	+
WG	47	M	MORTON'S NEUROMA	+
AK	33	M	CAUSALGIA, S/P SYMPATHETIC BLOCKS FOR RT.KNEE	+

TABLE IX-GROUP E

PATIENT	AGE	SEX	DIAGNOISIS	1bp13-14.719
BL	35	F	CERVICAL SPONDYLOSIS	+
RG	39	F	CERVICAL SPONDYLOSIS	+
CK	38	F	LIGAMENTOUS INJURY W/FLEXION DEFORMITY, C5-6	+

"+" indicates presence of 1bp13-14.719

the presence of lbp13-14.719 will not differentiate between these two conditions.

EXAMPLE 4

To determine whether there is a correlation between protein spot density and the severity of pain, the densities of the various proteins of interest in the initial study were compared to the degree of lower back disability, as scored by historical as well as physical and radiological determinations.

A short, abbreviated scoring system (abbreviated from a scale of 144 possible factors) was devised which is similar to the Waddell approach [Waddell and Main, "Assessment of severity in low-back disorders", Spine, Vol. 9, pp. 204-208 (1984); Waddell, Main, Morris, Paola and Gray, 1984; Waddell et al, 1980]. The physical signs which Waddell et al selected as being significant were: degree of lumbar flexion; straight leg raising; root compression signs; and previous lumbar surgeries. The six clinical signs used in the abbreviated scale were similar and constituted the clinical objective scale (COS):

1. Scar from previous back surgery. All back surgery creates permanent scarring with permanent changes, no matter how subtle. This contributes to the presence of minute to major back pain. Each surgery scored 2.

2. True spasm which the patient cannot control (the most significant sign of all). This is not a limitation of motion which the patient can control, but true, uncontrolled spasm which the physician can recognize. Although sometimes painless in a condition such as burned out ankylosing spondylitis, it otherwise invariably signals severe pain. The physician can easily recognize this exception. This finding scored 4.

3. Straight leg raising (SLR) (right and left). This must be differentiated from hamstring spasm and a functional SLR. Hamstring spasm or tightness produces pain locally in the thigh, not in the back with SLR. Functional SLR is easily recognized by having the patient sit on the edge of the table and casually extend

the knee. If the patient does not complain of back pain, there is no positive SLR. In knowledgeable hands, this is an objective finding. Each SLR scored 2.

4. Knee or ankle reflex change (right and left). These are objective findings in all cases. It is possible for them to be present without pain because of old trauma or surgery. Some reflex changes are associated with weakness or atrophy, but because these findings are frequently not recorded, it is not practical to score the latter. Each reflex change scored 1.

Table X summarizes the COS.

Table X

Summary of COS

FACTOR	VALUE
Each back incision (scar)	2
Spasm	4
Right Straight Leg Raising (RSLR)	2
Left Straight Leg Raising (LSLR)	2
Right Reflex Change (RRC)	1
Left Reflex Change (LRC)	1

The abbreviated scoring of the patients of this study are seen in Table XI, below. The numbers indicated next to the gel are arbitrarily assigned patient numbers.

Table XI. Scores of COS of Patients.

Patient	SCAR	SPASM	RSLR	LSLR	RRC	LRC	TOTAL
gel 1	2	4		2			8
gel 3				2			2
gel 5							0
gel 7	2		2				4
gel 9							0
gel 11			2	2	1		5
gel 13	2			2			4
gel 15	2	4	2	2		1	11
gel 17							0
gel 19	2			2		1	5

By comparing the above COS scores to the amount of protein 1318 in the gels, it was found that the protein designated 1318 displayed a correlation with pain severity. This is represented in Figure 5.

The same comparison was made with proteins 1316, 1204, 1305, 3203 and 3211. Also a comparison was made with the average of proteins 1318 and 1316. These results are shown in Figures 6-11.

EXAMPLE 5

To examine the effectiveness of a course of treatment for back pain, a blood sample would be obtained from a chronic back pain patient prior to the treatment. A two-dimensional gel would be run, stained and analyzed for levels of one or more of the proteins listed in Tables II and III to obtain a baseline. After instituting treatment, one or more blood samples are taken from the patient and analyzed for levels of the same protein or proteins which were initially analyzed. A proportional increase or decrease to normal levels (depending on whether the protein analyzed is one which is found to increase or decrease in chronic back pain patients) signifies that the treatment is successful.

EXAMPLE 6

A. Preparing Antigens. After two-dimensional gel electrophoresis is performed on a patient with confirmed chronic lower back pain, the gel would be stained with Coomassie blue in order to locate a protein of interest. The gel is rinsed with deionized water for a few minutes, changing the water several times. The spot containing a protein is cut out of the gel with a scalpel, and placed on a piece of parafilm or plastic wrap. The edge of a paper towel is used to remove any standing water by capillary action. Next, the plungers from the barrels of two 5 cc syringes are removed, and the gel piece is placed into one of the barrels. The plunger is then replaced and the syringe outlet is positioned in the barrel of the second syringe. Using rapid, firm pressure on the plunger, the gel is pushed into the barrel of the second syringe. This process is repeated several times back and forth between the two syringes. Then, 21-gauge needles are placed onto the outlet of the syringes, and the process is repeated. A small amount of buffer (PBS) may be necessary to keep the small fragments passing back and forth between the syringes. The samples are now ready for injection.

B. Preparing Antisera. Antibodies are raised in rabbits immunized by injecting the antigen preparation (above). An initial

subcutaneous injection of approximately 150 ug of one of the protein preparations would be followed by two monthly injections of approximately 100 ug of the antigen. This will lead to a sufficient antibody titer for use in an immunoassay.

C. Preparing Monoclonals. Monoclonal antibodies may be prepared according to the method of Köhler and Milstein. This method involves immunizing mice with an antigen bearing one or more epitopes (i.e., one of the lower back pain proteins). The mice develop spleen cells making anti-epitope(s) which appear as an antibody (or antibodies) in the serum. The spleen is removed and the individual cells fused in polyethylene glycol with constantly dividing (i.e., immortal) B-tumor cells selected for a purine enzyme deficiency and often for their inability to secrete Ig. The resulting cells are distributed into micro-well plates in HAT (hypoxanthine, aminopterin, thymidine) medium which kills off the perfusion partners, at such a high dilution that, on average, each well will contain less than one hybridoma cell. Each hybridoma being the fusion product of a single antibody-forming cell and a tumor cell will have the ability of the former to secrete a single species of antibody and the immortality of the latter enabling it to proliferate continuously, clonal progeny providing an unending supply of antibody.

D. Western Blot (Immunoblot). Proteins from the two-dimensional gels of Example 1 are electrophoretically eluted, prior to staining, to 0.2 um nitrocellulose membranes. The membranes are rinsed with PBS and incubated with BSA (3% BSA (fraction V, 0.02% sodium azide in PBS). The membranes are then incubated with primary rabbit polyclonal antibodies (obtainable by the above method) at a concentration of about between 1 and 50 ug/ml in PBS. The membranes are then washed with several changes of PBS, followed by incubation with goat anti-rabbit IgG labeled with horseradish peroxidase. Finally, after rinsing with PBS, the membranes are developed with 4-chloro-1-naphthol (stock solution is 0.3 g

chloronaphthol in 10 ml absolute ethanol; working solution is 0.1 ml stock added to 10 ml of 50 mM TRIS, pH 7.6; the white precipitate is filtered and 10 ul of 30% hydrogen peroxide is added). The reaction is stopped by rinsing with PBS. A positive result is seen if the spots of interest develop into a blue-black color.

In the foregoing immunoblot procedure, monoclonal antibodies may be substituted for the primary polyclonal antisera to obtain a higher specificity.

E. Radioimmunoassay (RIA). To perform this assay, one would use a monoclonal antibody to one of the proteins of interest (such as those exemplified in Example 1), which would be prepared according to known methods as discussed above.

A sheet of nitrocellulose paper is cut to the size of a dot blot apparatus. The sheet is pre-wetted with water, and fitted onto the apparatus. A plasma sample from the patient is placed in the wells (30 ul/well) in serial dilutions, and incubated for two hours in a humid atmosphere. The sheet is then washed with two changes of PBS. The sheet is then blocked by incubating with a solution of 3% BSA/PBS with 0.02% sodium azide for at least 2 hours. Following washing with PBS, the primary monoclonal antibody (in a solution of 3% BSA/PBS with 0.02% sodium azide) is added at a suitable dilution and incubated for 2 hours with agitation. Unbound antibody is washed away with PBS. An I^{125}-labeled goat anti-rabbit IgG (in 3% BSA/PBS with 0.02% sodium azide) is then incubated with the sheet for about 2 hours with agitation. Unbound labeled antibody is removed by washing four times with PBS for 5 minutes each. The amount of bound labeled antibody is determined by autoradiographic detection. This is done by placing the sample sheet in direct contact with an X-ray film and storing this system at -70°C with an intensifying screen. Results can be crudely quantitated by visual examination of the exposed film and more quantitatively by densitometric tracing. The relative amounts of antigen in different samples are determined by comparing midpoints

of the titration curves. Absolute amounts of antigen can be determined by comparing these values with those obtained using known amounts of antigen.

F. Enzyme-linked Immunosorbent Assay (ELISA). This immunoassay is performed as set forth above in the RIA method; however, rather than the secondary antibody, goat anti-rabbit IgG, being labeled with I^{125}, it is labeled with horseradish peroxidase (HRP). In order to detect and quantify the HRP, the dot blot is developed with chloronaphthol. 4-Chloro-1-naphthol (0.3 g) is dissolved in 10 ml of absolute ethanol to prepare a stock solution. Immediately prior to developing the assay, 0.1 ml of the stock is added to 10 ml of 50mM TRIS (pH 7.6). The white precipitate formed is filtered with Whatman No. 1 filter paper. 10 ul of 30% H_2O_2 is added to the solution. The chloronaphthol solution is added to the nitrocellulose sheet and agitated until the spots are suitably dark (about 30 min.). The reaction is stopped by rinsing with PBS. The results can be determined as with the RIA, and quantification performed by visual inspection or by reflection densitometry.

EXAMPLE 7

Typical test kits for use with RIA or ELISA tests will contain:

A

1. A plate with absorbed rabbit Fab fragment IgG (to any of the proteins set forth in Tables II, III and IV), or nitrocellulose sheets with the absorbed rabbit IgG.

2. Rabbit whole IgG (to the same protein as above).

3. Labeled goat anti-rabbit IgG.

B

1. Rabbit IgG (to any of the proteins listed in Tables II, III and IV).

2. Labeled goat anti-rabbit.

These kits may also contain appropriate buffers such as PBS, blocking solution, and appropriate enzyme substrates (for ELISAs).

These materials may be provided with the kit or may be separately provided or prepared.

The term "plate" is used in the broad sense to include any flat surface which can be employed with an RIA or ELISA.

In practice the test kit A (above) would be employed as follows:

1. Incubate the plate with the serum of the patient under test for an appropriate time and temperature (e.g., from 2-4 hours at $37^{O}C$).

2. Wash with BSA/PBS.

3. Incubate with rabbit whole IgG and wash with buffer.

4. Incubate with labeled goat anti rabbit IgG and wash with the same buffer.

5. Detect the formation of a reaction product (or radioactive signal) in the case of a positive test by any of the aforementioned procedures.

The test kit B would be employed as follows:

1. Incubate a substrate (plate, nitrocellulose paper, etc.) with an unknown sample (such as plasma) for an appropriate time and temperature.

2. Wash with BSA/PBS.

3. Incubate substrate with rabbit IgG and wash with buffer.

4. Incubate with goat anti-rabbit IgG and wash with same buffer.

5. Detect the formation of a reaction product (or radioactive signal) in the case of a positive test by an appropriate procedure.

We claim:

1. A method of diagnosing chronic back or cervical pain, comprising:

subjecting a body fluid sample of a patient suspected of having chronic back or cervical pain to two-dimensional electrophoresis or an immunoassay; and

measuring a protein or proteins which increase or decrease in concentration, as compared to control samples from individuals without chronic back or cervical pain.

2. The method of claim 1, wherein the body fluid sample comprises plasma or serum.

3. The method of claim 2, wherein said plasma is subjected to two-dimensional electrophoresis.

4. The method of claim 3, wherein said proteins are detected by silver-staining or staining with Coomassie blue.

5. The method of claim 4, further comprising quantifying the concentration by measuring density with a densitometer.

6. The method of claim 1, wherein said proteins which increase in concentration comprise at least one selected from the group consisting of: 4210, 1204, 1318, 1324, 1702, 4821, 0508, 3211, 4109, 5524, 7622, 6331, 8322, 3151, 1323, 5240, 2105, 1316, 6104, 7109, 3606, 1305, 3146, 7114, 7448, 3203, 0609, 0604, 7711 and 5615 as identified in Figure 1.

7. The method of claim 1, wherein said protein which increases in density is lbp13-14.719.

8. The method of claim 6, wherein said proteins which increase in concentration comprise at least one selected from the group consisting of: 1316, 1324, 1204, 1305, 1318 and 1323.

9. The method of claim 1, wherein said proteins which decrease in concentration comprise at least one selected from the group consisting of 4737, 4624, 7212, 7309, 7214, 2806, 5013, 4614, 3155, 8309, 8418, 7455, 5411 and 7515 as identified in Figure 1.

10. A method of diagnosing chronic back pain, comprising:

subjecting a body fluid sample from a patient suspected of having chronic back pain to two-dimensional electrophoresis or an immunoassay; and

detecting the presence of at least one protein selected from the group consisting of: 4210, 1204, 1318, 1324, 1702, 4821, 0508, 3211, 4109, 5524, 7622, 6331, 8322 and lbp13-14.719, wherein the presence of said at least one protein confirms that said patient has chronic lower back pain.

11. The method of claim 10, wherein the body fluid sample comprises plasma or serum.

12. The method of claim 11, wherein said plasma is subjected to two-dimensional electrophoresis.

13. The method of claim 12, wherein said protein or proteins are detected by silver-staining or staining with Coomassie blue.

14. The method of claim 12, wherein said protein or proteins are detected by a western blot technique.

15. A method of diagnosing chronic back pain, comprising:

subjecting a body fluid sample from a patient suspected of having chronic back pain to two-dimensional electrophoresis or an immunoassay; and

detecting the presence or absence, as compared to a control sample from an individual without chronic back pain, of at least one protein selected from the group consisting of: 4737, 4624, 7212, 7309, 7214, 2806 and 5013, wherein the absence of said

at least one protein confirms that said patient has chronic lower back pain.

16. The method of claim 15, wherein the body fluid sample comprises plasma or serum.

17. The method of claim 16, wherein said plasma is subjected to two-dimensional electrophoresis.

18. The method of claim 17, wherein the absence of said protein or proteins is detected by silver-staining or staining with Coomassie blue.

19. The method of claim 15, wherein the absence of said protein or proteins is confirmed by a western blot technique.

20. A method of determining the severity of chronic lower back pain, comprising:

subjecting a body fluid sample from a patient with chronic back pain to two-dimensional electrophoresis or an immunoassay;

determining the concentration of at least one protein selected from the group consisting of: 1318, 1204, 1305, 1316, 3203 and 3211; and

comparing said concentration to a predetermined clinical correlation table,

wherein the degree of increase of the protein or proteins determines the severity of chronic lower back pain as compared to said table.

21. The method of claim 20, wherein the severity of chronic lower back pain is determined by measuring the increase of an average concentration of proteins 1318 and 1316.

22. The method of claim 20, wherein the body fluid sample comprises plasma or serum.

23. The method of claim 22, wherein said plasma is subjected to two-dimensional electrophoresis.

24. The method of claim 23, wherein the concentration of the protein or proteins is determined by silver-staining or staining with Coomassie blue followed by densitometric analysis.

25. A method of determining the effectiveness of treatment for chronic back or cervical pain, comprising:

measuring the concentration of a protein or proteins in a body fluid sample from a patient with chronic back or cervical pain prior to initiating treatment; and

measuring the concentration of the same protein or proteins of a same body fluid sample after treatment has been instituted,

wherein a proportional decrease or increase of protein to levels found in an individual without chronic back or cervical pain indicates the effectiveness of treatment.

26. The method of claim 25, wherein the body fluid sample comprises plasma or serum.

27. The method of claim 26, wherein said plasma is subjected to two-dimensional electrophoresis or an immunoassay.

28. The method of claim 27, wherein the two-dimensional gels are stained by silver-staining or staining with Coomassie blue.

29. The method of claim 28, further comprising quantifying the concentration by measuring the stain density with a densitometer.

30. A method of diagnosing cervical spine pain, comprising subjecting a body fluid sample from a patient suspected of having cervical spine pain to two-dimensional electrophoresis or an immunoassay; and

detecting the presence of lbp13-14.719, wherein the presence of lbp13-14.719 confirms that said patient has cervical spine pain.

31. An antibody to one of the proteins selected from the group consisting of: 4210, 1204, 1318, 1324, 1702, 4821, 0508, 3211, 4109, 5524, 7622, 6331, 8322, 3151, 1323, 5240, 2105, 1316, 6104, 7109, 3606, 1305, 3146, 7114, 7448, 3203, 0609, 0604, 7711, 5615, 4737, 4624, 7212, 7309, 7214, 2806, 5013, 4614, 3155, 8309, 8418, 7455, 5411, 7515 and lbp13-14.719.

32. The antibody of claim 30 which is obtained from polyclonal antibodies present in antisera.

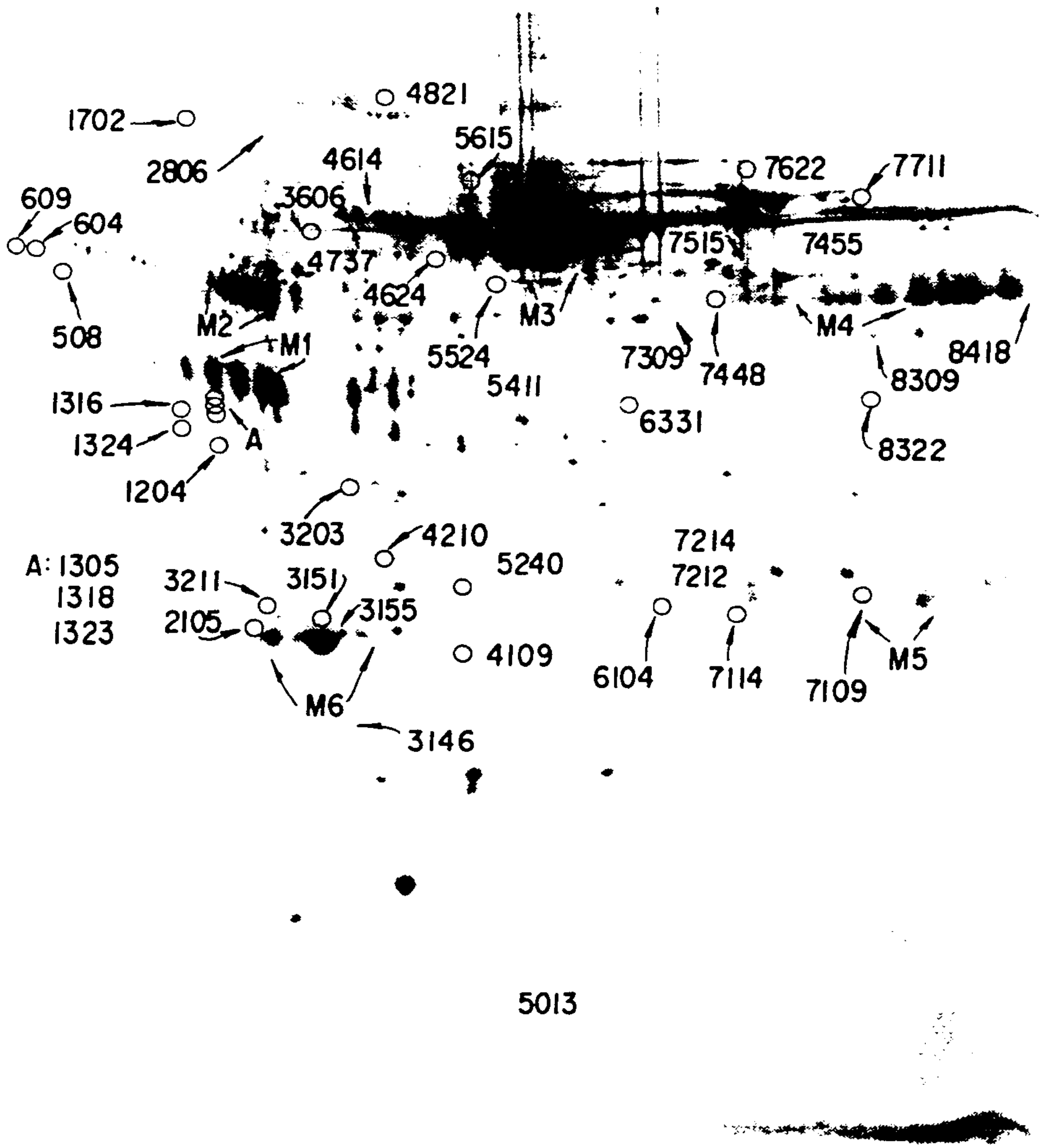
33. The antibody of claim 30 which is a monoclonal antibody.

34. A test kit for detecting chronic lower back or cervical pain, said kit comprising:

a diagnostically effective amount of an antibody to one of the proteins selected from the group consisting of: 4210, 1204, 1318, 1324, 1702, 4821, 0508, 3211, 4109, 5524, 7622, 6331, 8322, 3151, 1323, 5240, 2105, 1316, 6104, 7109, 3606, 1305, 3146, 7114, 7448, 3203, 0609, 0604, 7711, 5615, 4737, 4624, 7212, 7309, 7214, 2806, 5013, 4614, 3155, 8309, 8418, 7455, 5411, 7515 and lbp13-14.719; and

a labeled antibody to said antibody.

35. The test kit of claim 33, wherein said antibody is polyclonal or monoclonal.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M4
M1
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7448
8418
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6331
8322
1324
A
1204
3203
4210
7214
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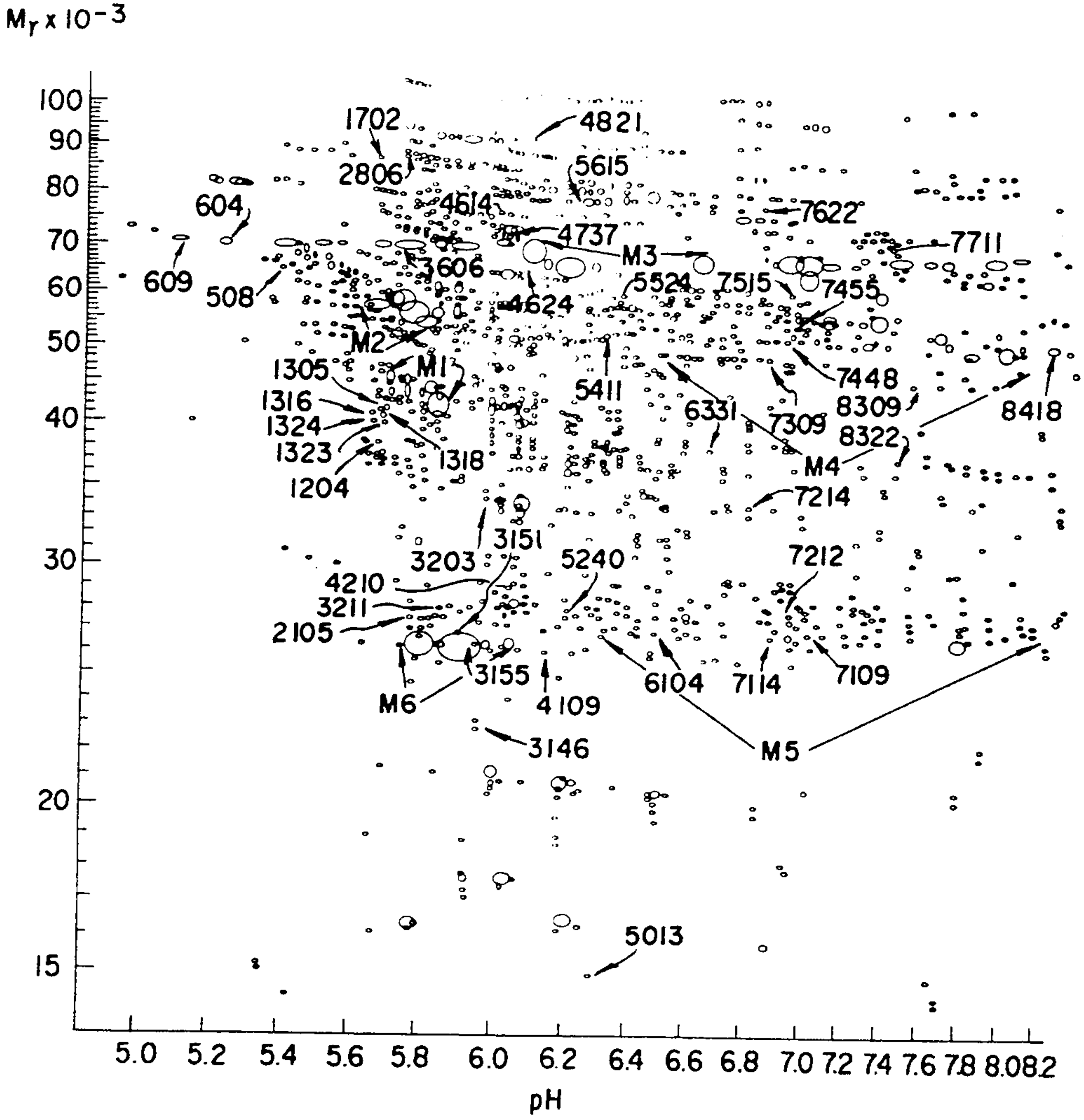
FIG. 1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5524
7515
7455
4624
M2
M1
1305
5411
7448
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6331
8309
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1324
7309
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8322
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M4
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7214
3151
7212
3203
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7114
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4109
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5.6
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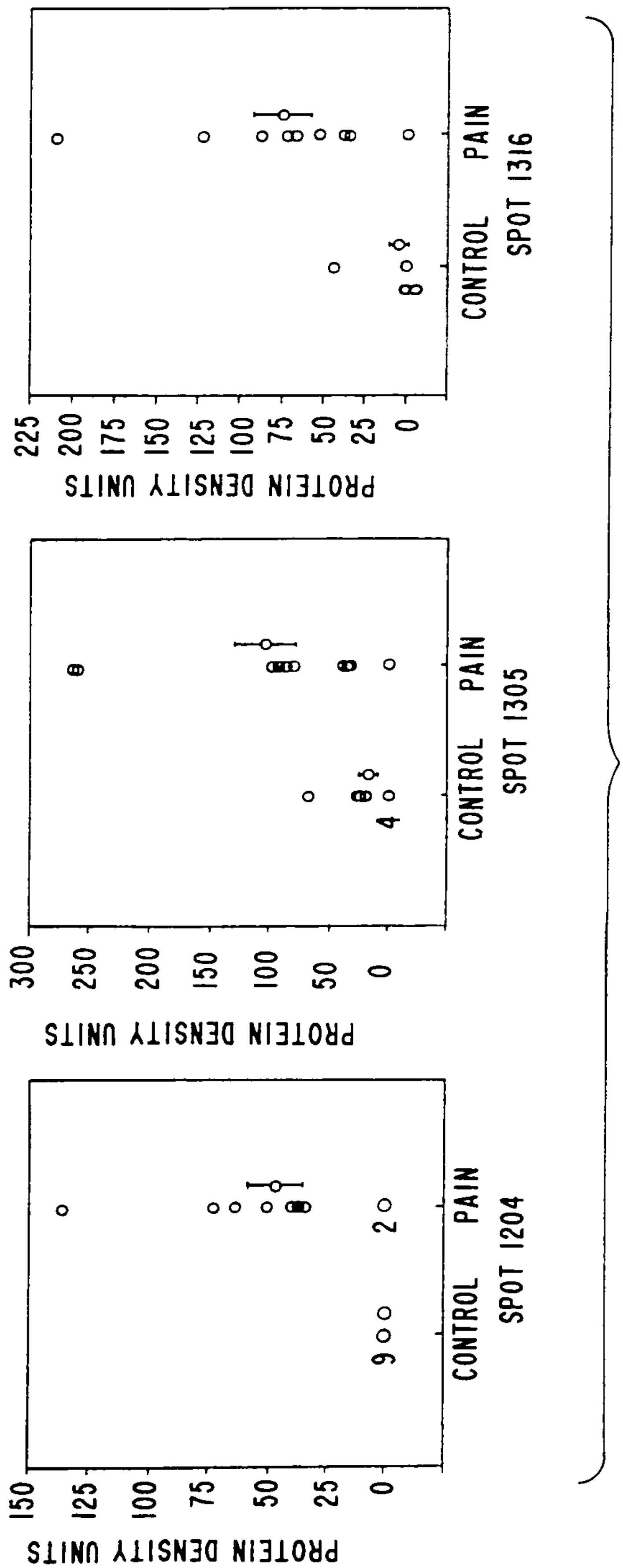
8.2
pH

FIG. 2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CONTROL PAIN
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PROTEIN DENSITY UNITS
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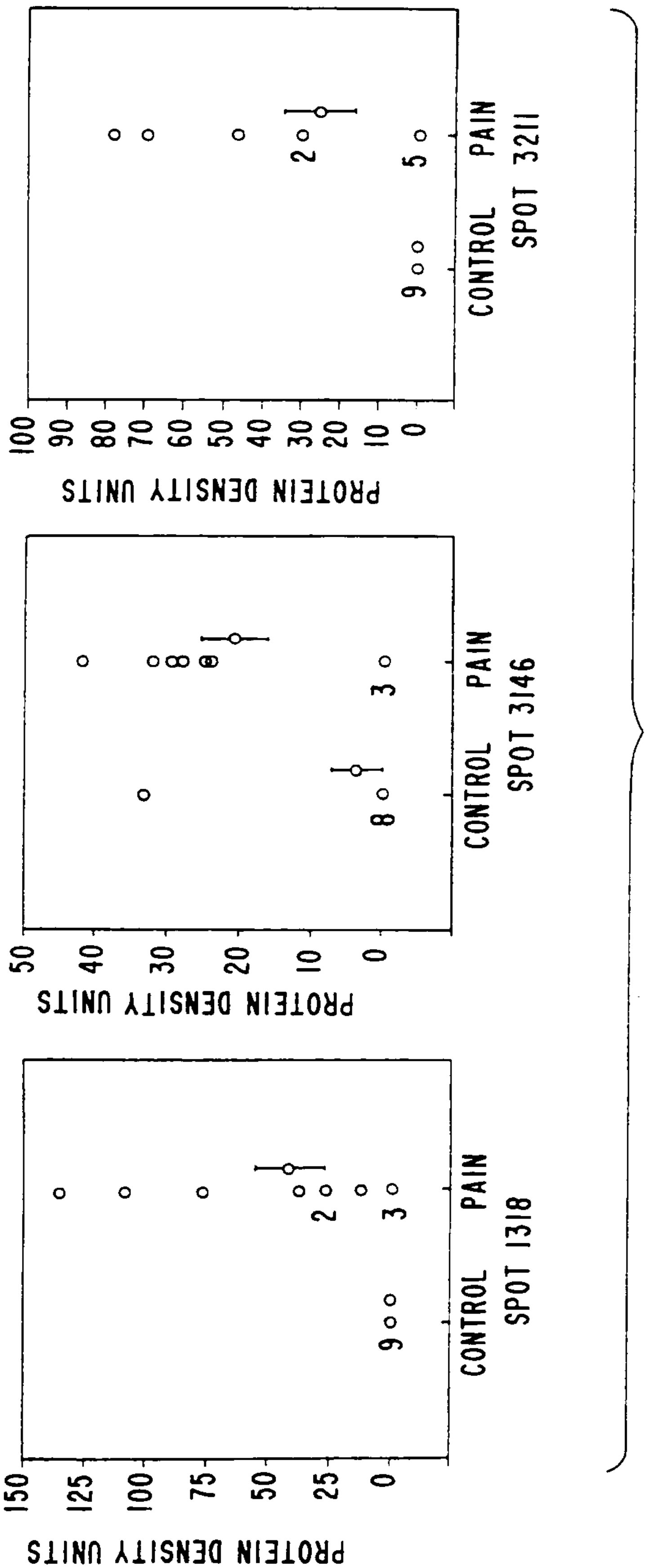
CONTROL PAIN
SPOT 1204

Fig.3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0
10
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30
40
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CONTROL PAIN
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3

Fig.4

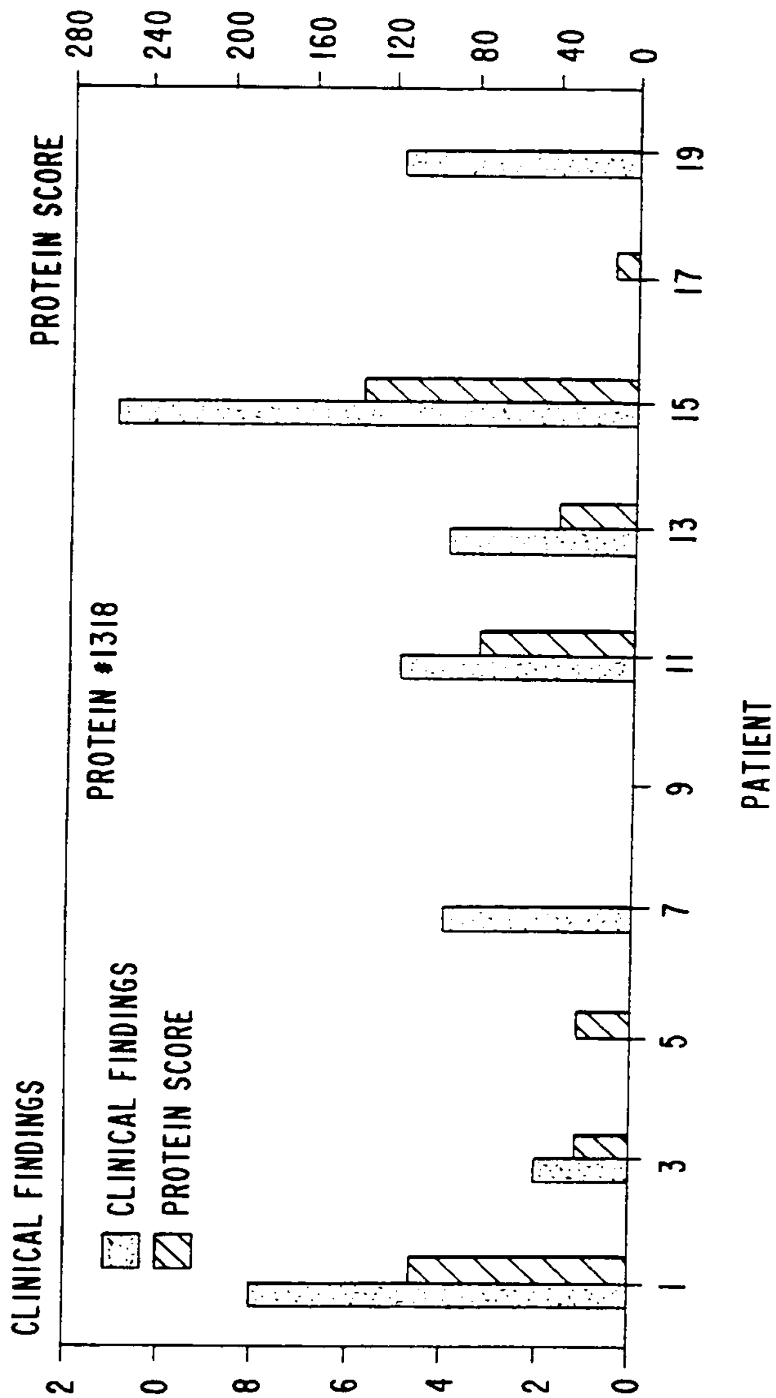
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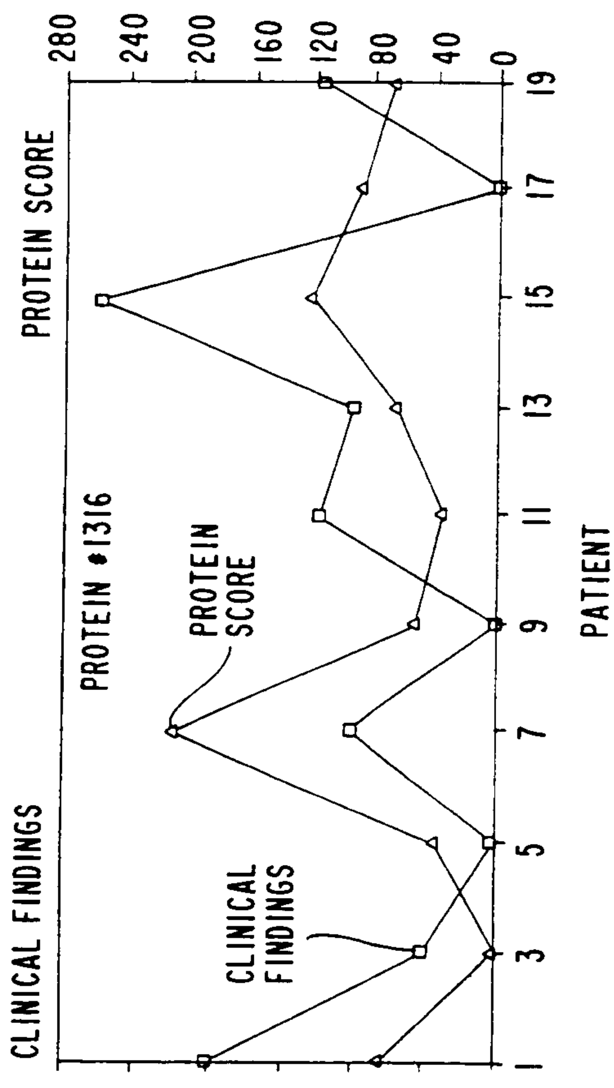
Fig.5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17
19

Fig.6

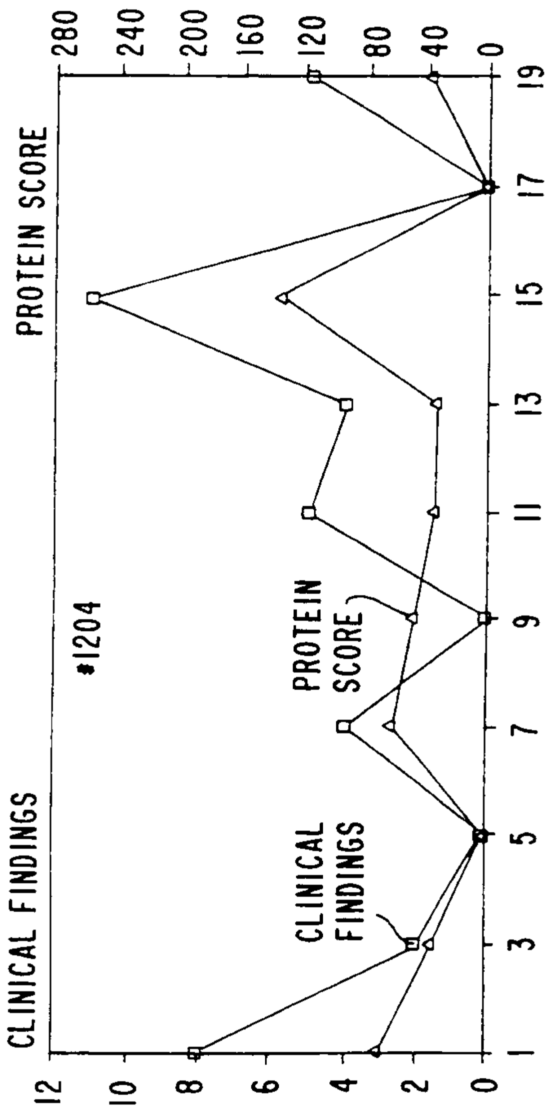
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Fig.7

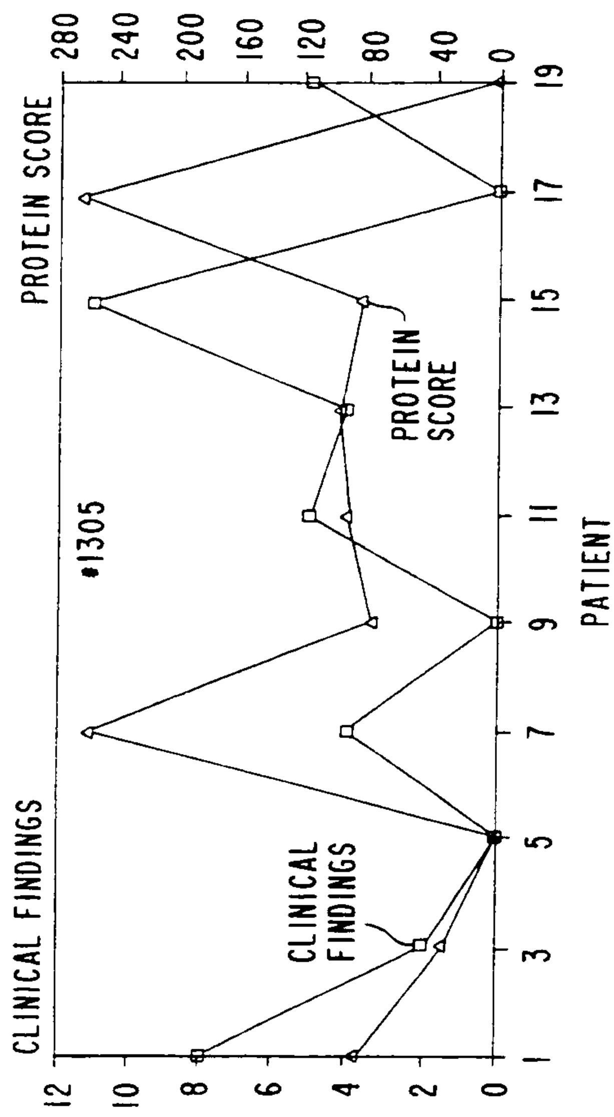
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Fig.8

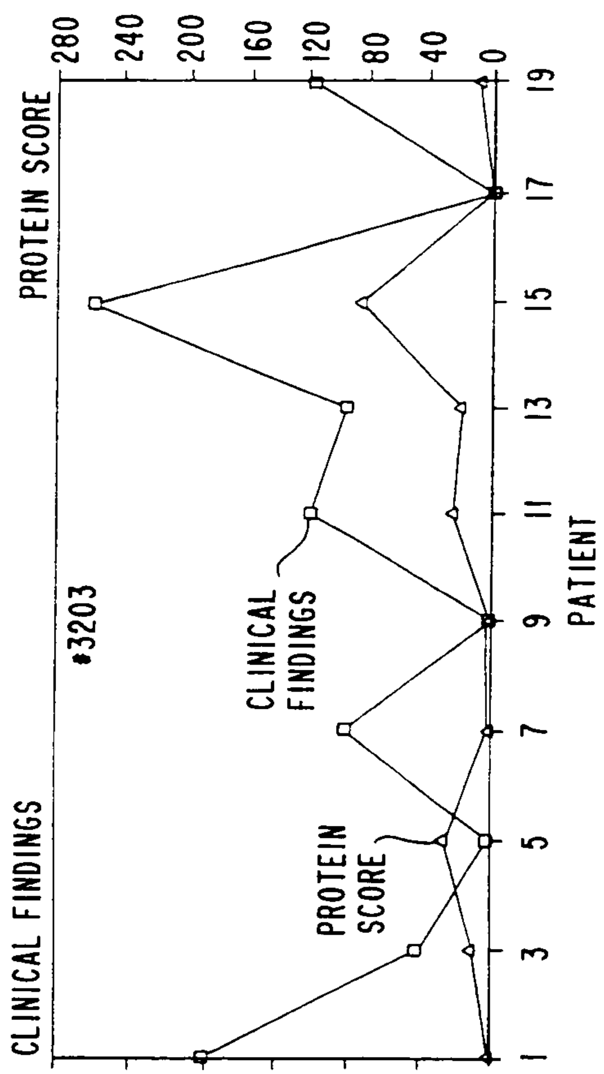
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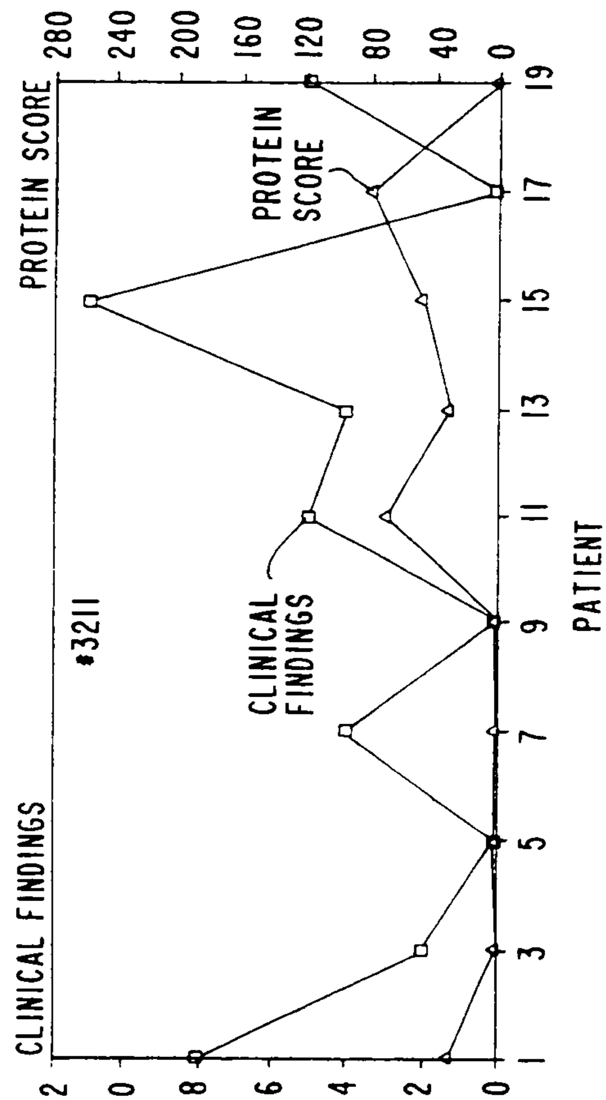
Fig.9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PROTEIN SCORE
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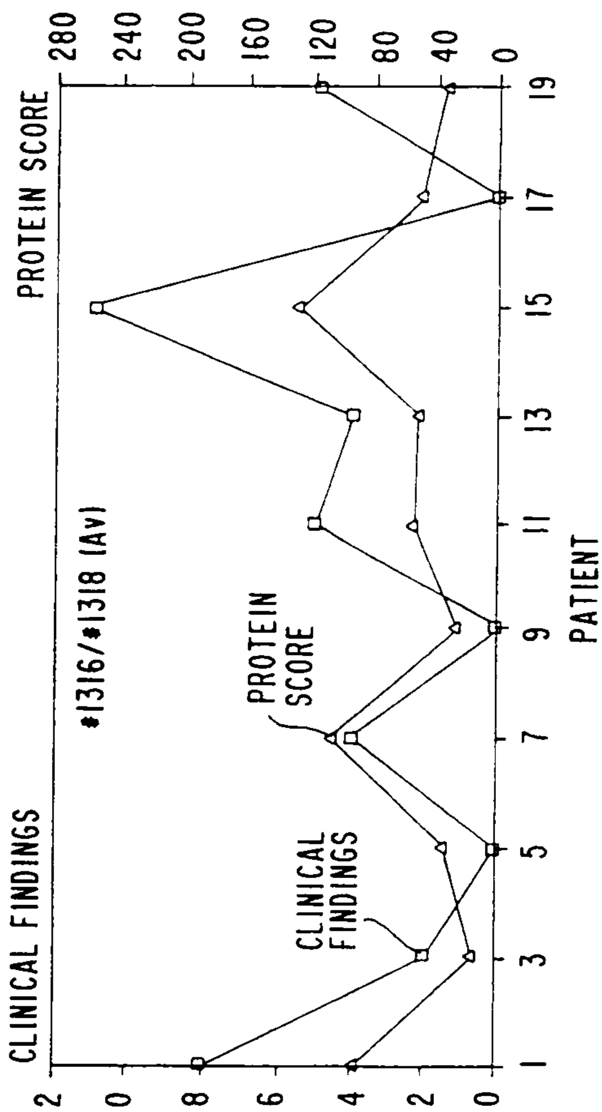
1 3 5 7 9 11 13 15 17 19

Fig.10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PROTEIN SCORE
CLINICAL FINDINGS
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13
15
17
19

Fig.11

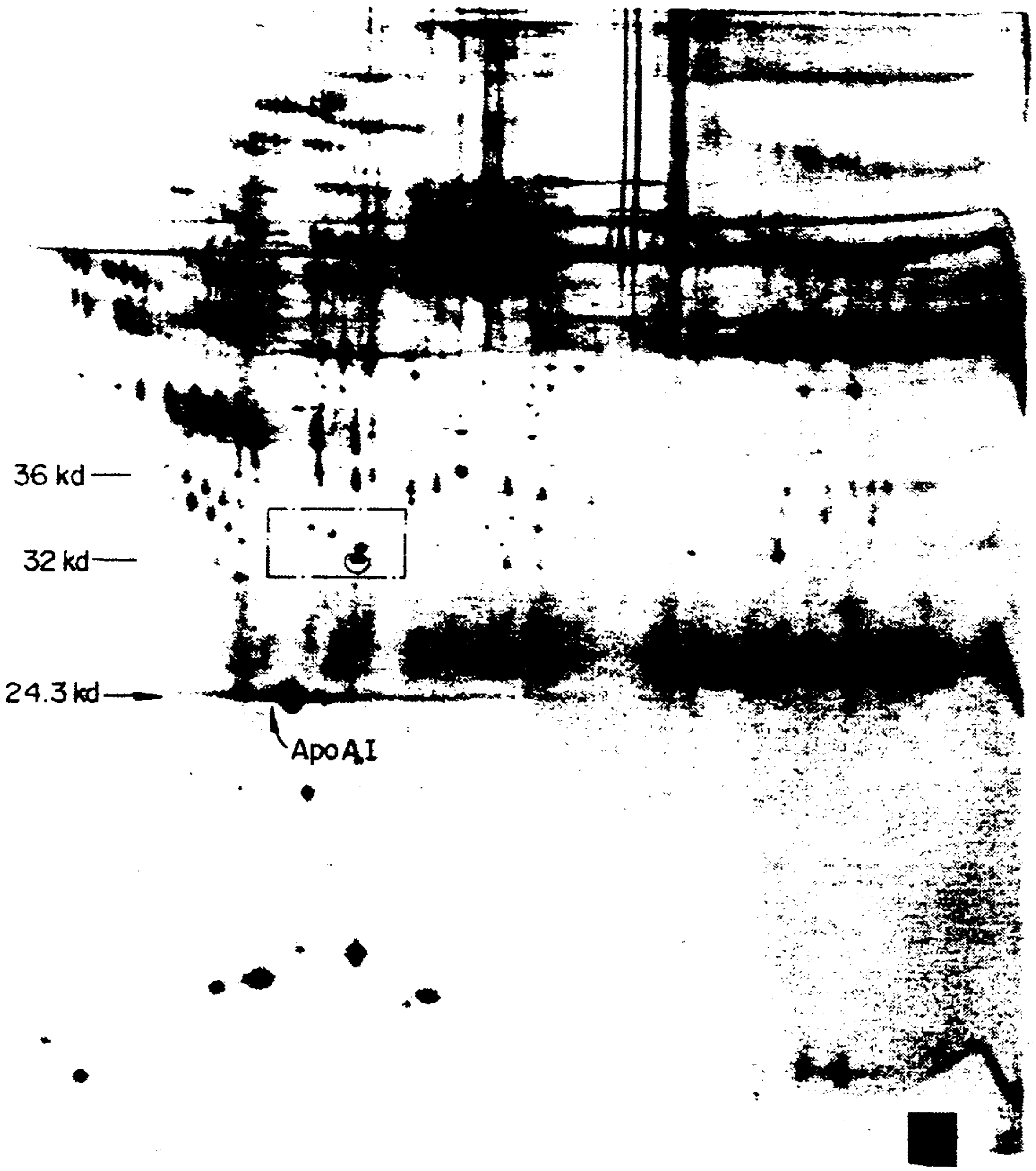

FIG. 12

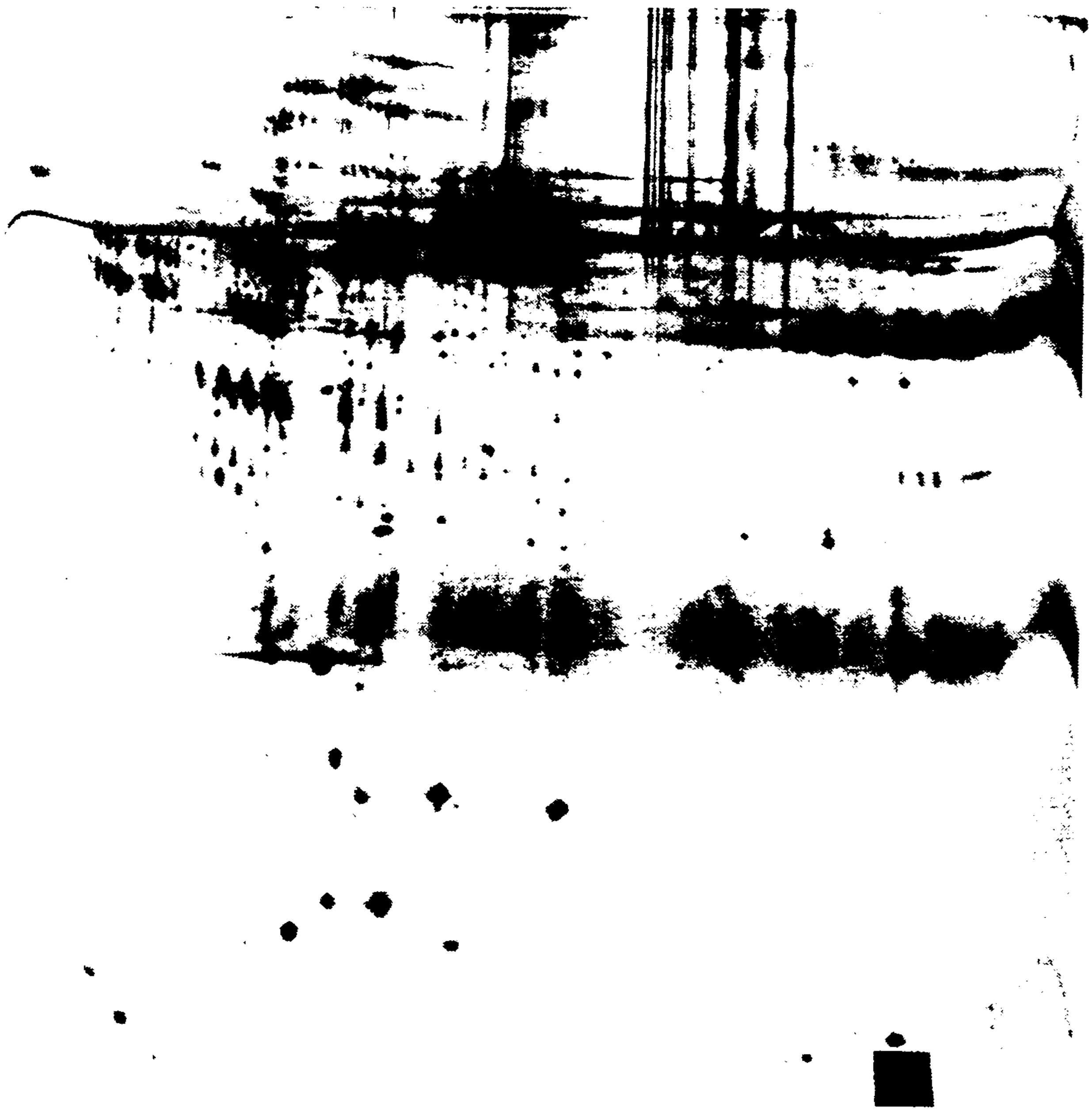

FIG. 13

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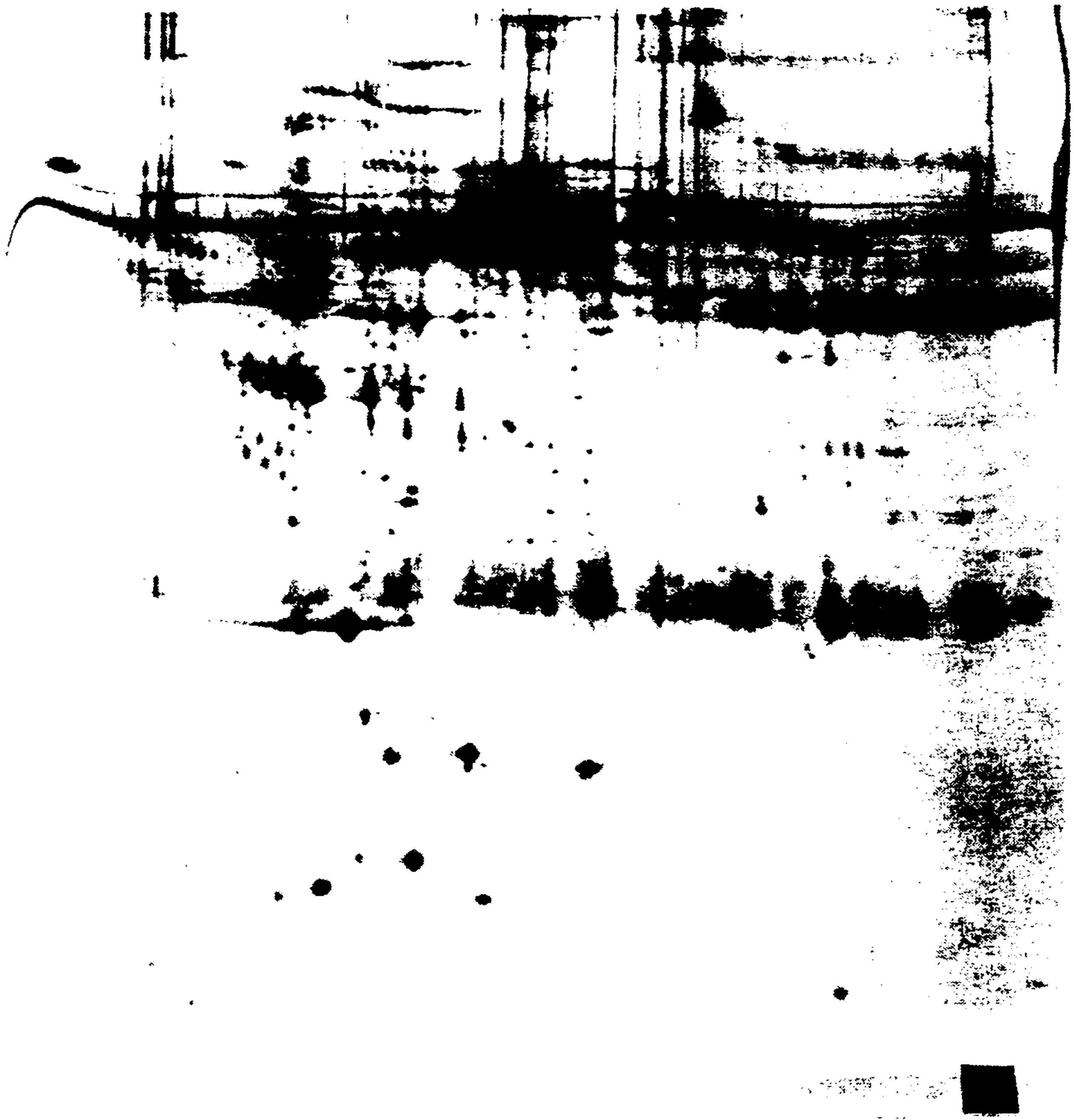

FIG. 14

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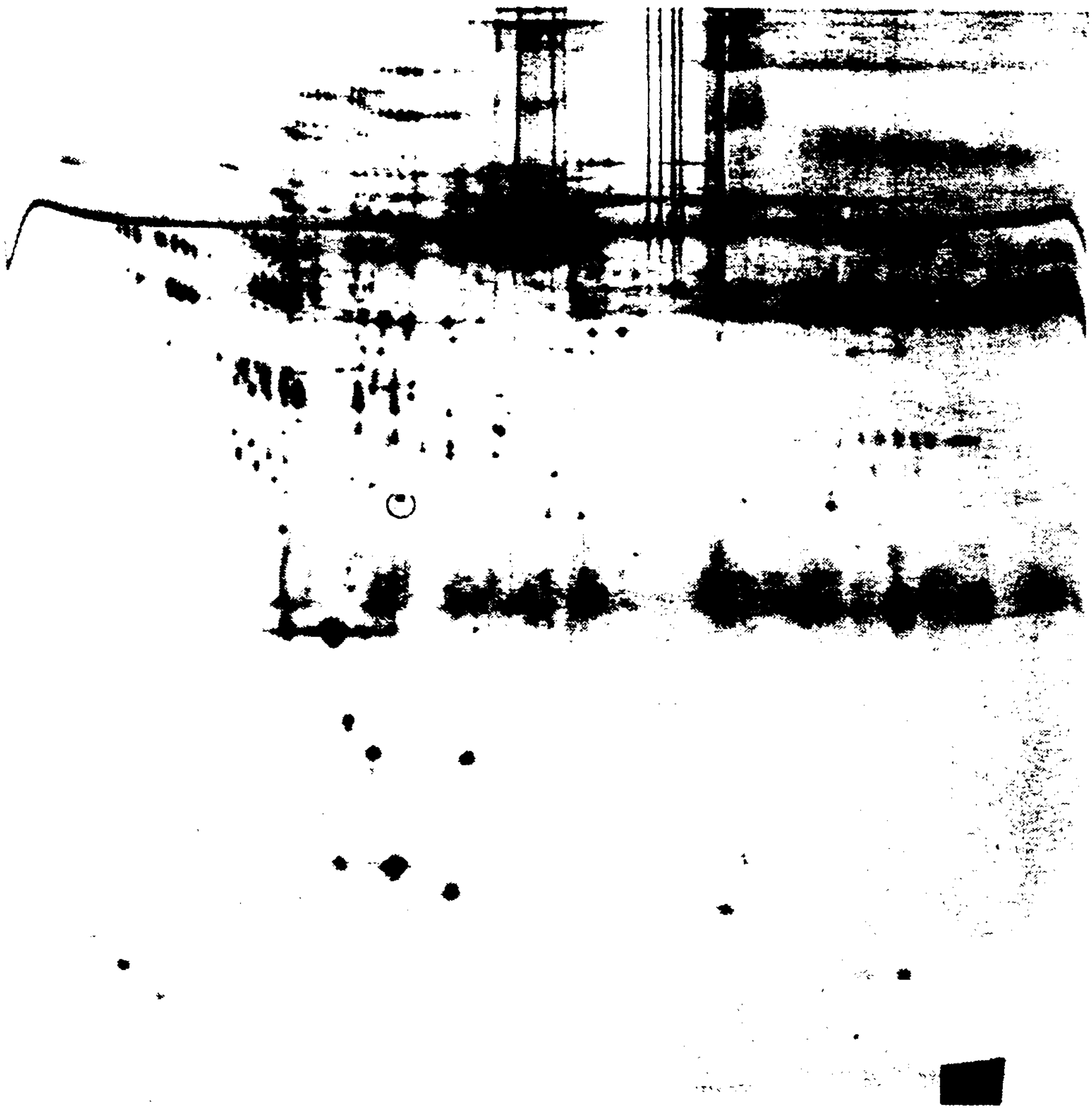

FIG. 15

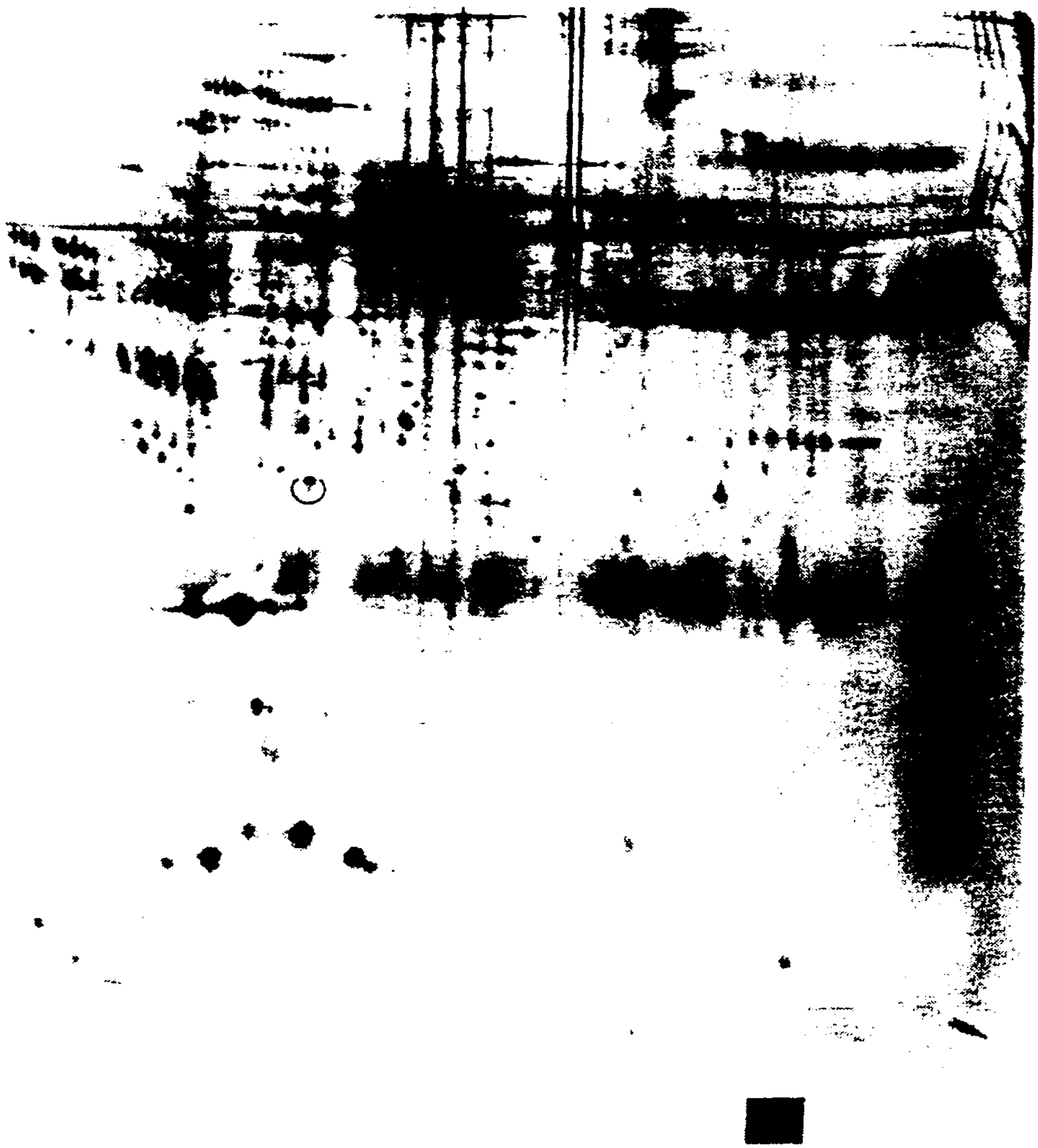

FIG. 16

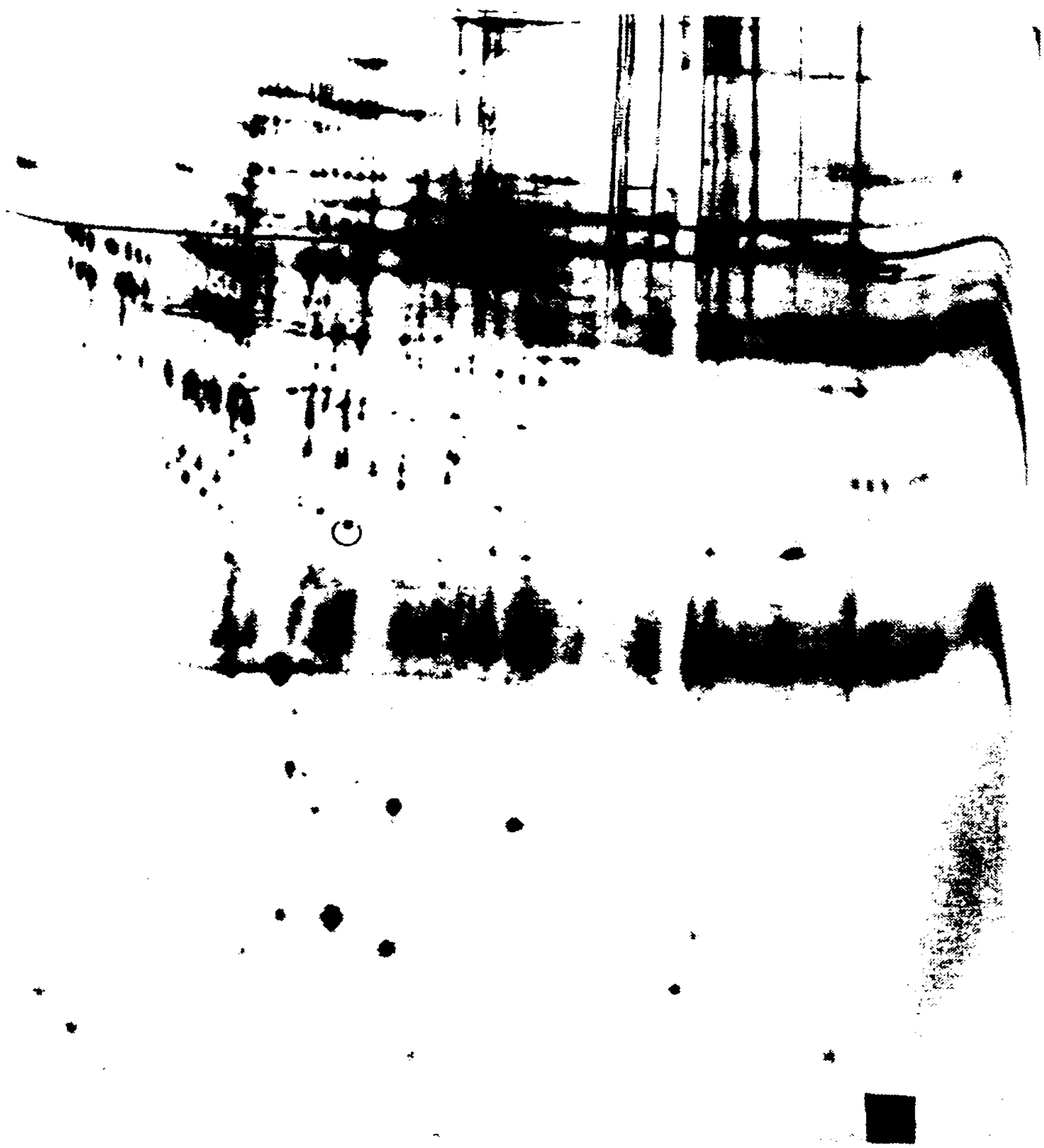

FIG. 17

SUBSTITUTE SHEET

FIG. 18

FIG. 19

FIG. 20

SUBSTITUTE SHEET

FIG. 23

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/08552

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/68; G01N33/561

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	ACTA NEUROLOGICA SCANDINAVICA vol. 58, no. 6, 1978, COPENHAGEN pages 358 - 365; A. AHONEN ET AL: 'Measurement of reference values for certain proteins in cerebrospinal fluid.' see the whole document ---	1-35
Y	THE JOURNAL OF CLINICAL LABORATORY AUTOMATION vol. 3, no. 4, 1 July 1983, NEW YORK NY USA pages 235 - 243; R. P. TRACY ET AL.: 'Two-dimentional gel electrophoresis : methods and potential applications in the clinical laboratory.' cited in the application see page 239, column 1, line 8 - page 240, column 2, line 15 ---	1-35

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2 07 APRIL 1992	14.04.92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	VAN BOHEMEN C.G. C.G. van Bohemen

Form PCT/ISA/210 (second sheet) (January 1985)

FIG. 21

FIG. 22